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(54) Title: PROMOTER SEQUENCE FROM POTATO

(57) Abstract

A promoter is described that is capable of expressing a GOI in at least any one of root, tuber, sprout and tuber tissue of a dicot plant. In particular, the promoter is a promoter for alpha-amylase.

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Promoter Sequence From Potato

The present invention relates to a promoter, including a construct and an expression vector comprising the same and a transformed cell comprising the same. In addition the present invention relates to a plant comprising the same.

It is known that it is desirable to direct expression of a gene of interest ("GOI") in certain tissues of an organism – such as a plant. For example, it may be desirable to produce crop protein products with an optimised amino acid composition and so increase the nutritive value of the crop. It may even be desirable to use the crop to express non-plant genes such as genes for mammalian products. Examples of the latter products include interferons, insulin, blood factors and plasminogen activators.

However, whilst it may be desirable to achieve expression of a GOI in certain tissues it is sometimes important (if not necessary) to ensure that the GOI is not expressed in other tissues in such a manner that detrimental effects may occur. Moreover, it is important not to upset the normal metabolism of the organism to such an extent that detrimental effects occur. For example, a disturbance in the normal metabolism in a plant's leaf or root tip could lead to stunted growth of the plant.

CA-A-2006454 describes a DNA sequence of an expression cassette in which the potato tuber specific regulatory regions are localised. The expression cassette contains a patatin-gene with a patatin-gene promoter. The DNA sequence is transferred into a plant genome using *agrobacteria*. According to CA-A-2006454, the DNA sequence enables heterologous products to be prepared in crops.

One of the key plant enzymes is α-amylase. α-amylase participates in the pathway responsible for the breakdown of starch to reducing sugars in potato tubers. Genes coding for α-amylase in potato plants have been isolated and characterised. For example, see the teachings in EP-B-0470145.

In brief, α-amylase is encoded by a gene family consisting of at least 5 individual genes. Based on their homology the genes can be divided into two subfamilies – one of which is the type 3 amylase(s), the other of which is the type 1 amylase(s). The two groups of α-amylases are expressed differently, not only on the molecular level but also in different tissues of the potato plant.

In this regard, type 3 α-amylases are expressed in root, in tubers, in sprouts and in stem tissue; whereas type 1 α-amylases are expressed in sprout and stem tissues, but not in tubers.

The present invention seeks to provide a plant promoter that is capable of directing the expression of a gene of interest in specific tissues, or in just a specific tissue, of an organism, typically a plant.

According to a first aspect of the present invention there is provided a promoter comprising a nucleotide sequence corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof.

A restriction map of the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum* is shown in Figures 1, 2 and 8 – which are discussed later.

According to a second aspect of the present invention there is provided a promoter comprising a nucleotide sequence corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof but wherein at least a part of the promoter is inactivated.

According to a third aspect of the present invention there is provided a promoter comprising at least the nucleotide sequence shown as Seq.I.D. No. 1 or a variant, homologue or fragment thereof.

According to a fourth aspect of the present invention there is provided a promoter comprising the nucleotide sequence of any of one of the sequences shown as

Seq.I.D.No.s 4 – 17, preferably any of one of the sequences shown as Seq.I.D.No.s 4 – 16, or a variant, homologue or fragment thereof.

According to a fifth aspect of the present invention there is provided a promoter comprising a nucleotide sequence corresponding to the 5.5. Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof, but wherein at least the nucleotide sequence shown as Seq.I.D. No. 1 is inactivated.

According to a sixth aspect of the present invention there is provided a promoter comprising a nucleotide sequence corresponding to the 5.5. Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof, but wherein at least any of one of the sequences shown as Seq.I.D.No.s 2 – 16 is inactivated.

According to a seventh aspect of the present invention there is provided a construct comprising the promoter according to the present invention fused to a GOI.

According to an eighth aspect of the present invention there is provided an expression vector comprising the promoter according to the present invention.

According to a ninth aspect of the present invention there is provided a transformation vector comprising the promoter according to the present invention.

According to a tenth aspect of the present invention there is provided a transformed cell comprising the promoter according to the present invention.

According to an eleventh aspect of the present invention there is provided a transgenic organism comprising the promoter according to the present invention.

According to a twelfth aspect of the present invention there is provided the use of the promoter according to the present invention as a cold inducible promoter.

According to a thirteenth aspect of the present invention there is provided a construct comprising the promoter of the present invention and a nucleotide sequence coding for anti-sense alpha-amylase.

According to a fourteenth aspect of the present invention there is provided the use of a promoter according to the present invention for expressing a GOI in tuber and/or sprout and/or root and/or stem of a plant, preferably in just or at least tuber of a plant.

Other aspects of the present invention include methods of expressing or transforming any one of the expression vector, the transformation vector, the transformed cell, including *in situ* expression within the transgenic organism, as well as the products thereof. Additional aspects of the present invention include uses of the promoters for expressing GOIs *in vitro* (e.g. in culture media such as a broth) and *in vivo* (e.g. in a transgenic organism).

Preferably, in any one of the expression vector, the transformation vector, the transformed cell or the transgenic organism the promoter is present in combination with at least one GOI.

Preferably the transformation vector is derived from *agrobacterium*.

Preferably the promoter is stably incorporated within the transgenic organism's genome.

Preferably the transgenic organism is a plant. Preferably the plant is a dicot plant.

More preferably, the plant is a potato plant.

An advantage of the present invention is that a promoter corresponding to the 5.5. Kb *EcoR*1 fragment isolated from *Solanum tuberosum* is able to direct expression of a GOI in any one of root, tuber, sprout and stem tissue of a dicot, for example, a potato. The same is true for the variant, homologue or fragment thereof.

Further surprising however is the fact that at least a part of the promoter sequence can be inactivated (e.g. truncated) and it can still express a GOI.

More surprising is the fact that the partially inactivated (e.g. truncated) promoter sequences can direct expression of a GOI in one or more specific tissues, such as just tuber tissue, rather than in combination of root, tuber, sprout and stem tissues.

In this regard, it was found that modified promoters corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum* containing inactivated nucleotide sequences upstream (i.e. towards the 5' end) of position −691 with reference to Figure 3 do not yield expression in any one of root, tuber, sprout or stem tissue. Examples of such modified promoters include modified promoters containing only nucleotide sequences downstream of position −692, such as the promoter sequences SEQ. I.D. No.s 2−3.

However, it was found that promoters corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum* containing inactivated nucleotide sequences upstream of position −1535 (with reference to Figure 3) yield expression only in tuber tissue. Examples of these types of promoters include those that contain only nucleotide sequences downstream of position −1535 but wherein they contain at least nucleotide sequences upstream of −691 (with reference to Figure 3), such as the promoter sequences SEQ. I.D. No.s 4−17, in particular SEQ. I.D. No.s 6−17, more in particular SEQ. I.D. No.s 6−16.

Moreover, it was found with the last type of promoters that if those promoters contained at least Seq. I.D. No. 1 high expression yields were observed in tuber tissue.

Thus preferred examples of promoter sequences for tuber specific expression of a GOI containing at least the sequence shown as Seq. I.D. 1 include those sequences shown as Seq. I.D. No.s 4 − 17, more preferably those sequences shown as Seq. I.D. No.s 6−17, even more preferably those sequences shown as Seq. I.D. No.s 6−16.

Furthermore, it was found that promoters corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum* containing inactivated nucleotide sequences downstream of position −1535 (with reference to Figure 3) yield expression in root and/or sprout and/or stem tissue. Examples of these types of promoters include those that contain only nucleotide sequences upstream of position −1535 (with reference to Figure 3).

Moreover, it was found that promoters corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum* containing an inactive Seq. I.D. No. 1 yield expression only in root and/or sprout and/or stem tissue. Examples of these types of promoters include those that do not contain Seq. I.D. No. 1.

Particularly preferred sequences are Seq. I.D. No.s 4 - 16.

Tissue specific expression, such as tuber specific expression, is particularly advantageous for a number of reasons.

First, a GOI (as defined below) can be expressed in a specific tissue type. This is particularly advantageous if the GOI is an anti-sense endogenous for the organism in question because expression of the anti-sense sequence in other tissues can be detrimental.

Second, it is possible to express a GOI coding for an agent giving the organism resistance against a disease associated with specific tissue(s). For example, the GOI may be a toxin against common scab - which normally affects tuber tissue.

Third, large quantities of the product of expression of a GOI where the GOI is, for example, a desired compound of benefit to humans or animals (e.g. a desirable foodstuff or an enzyme having a beneficial pharmaceutical effect) can be achieved. Furthermore, that product is easily retrievable.

Fourth, use of the promoter according to the present invention enables one to express a suitable nucleotide in order to change the organism's metabolism at a specific site – such as increasing starch levels in tuber or even producing modified starch therein.

A further surprising advantage is that the promoter of the present invention, in particular the promoter of the first aspect of the present invention, is cold-inducible – i.e. leads to expression in conditions of about from 0°C to 12° C, to about 4°C. Thus this promoter is very useful for expressing GOI's in conditions that would be of some benefit in cold conditions – in particular such as expression of the alpha-amylase gene (or active fragment thereof) of EP-B-0470145 (shown as SEQ. I.D. No: 18). More preferably the GOI is a nucleotide sequence that is anti-sense to that alpha-amylase gene (or active fragment thereof), such as that shown as SEQ.I.D. No: 19.

Highly preferred embodiments of each of the aspects of the present invention do not include the native promoter in its natural environment.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression. The promoters of the present invention are capable of expressing a GOI. In addition to the nucleotide sequences described above, the promoters of the present invention could additionally include conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the GOI. For example, suitable other sequences include the *Sh1*-intron or an ADH intron. Other sequences include inducible elements – such as temperature, chemical, light or stress inducible elements. Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' leader sequence (see Sleat Gene 217 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97). The promoter of the present invention may also be called Amy 3 promoter or Amy 351 promoter or alpha-Amy 351 promoter or alpha-Amy 3 promoter.

In addition the present invention also encompasses combinations of promoters or elements.

For example, a promoter of the present invention, such as a tuber specific promoter (see above), may be used in combination with a stem specific promoter (see above). Other combinations are possible. For example, the promoter of the present invention, such as a stem or tuber specific promoter, may be used in combination with a root promoter and/or a leaf promoter.

The term "corresponding" in relation to the present invention means that the promoter sequence need not necessarily be derived from *Solanum tuberosum*. For example, the promoter could be prepared synthetically. It may even be derived from another source.

The terms "variant", "homologue" or "fragment" include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to the sequence providing the resultant nucleotide sequence has the ability to act as a promoter in an expression system - such as the transformed cell or transgenic organism according to the present invention. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence has the ability to act as a promoter. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology, more preferably at least 95%, more preferably at least 98% homology.

The term "inactivated" means partial inactivation in the sense that the expression pattern of the complete promoter of Figure 8 is modified but wherein the partially inactivated promoter still functions as a promoter. However, as mentioned above, the modified promoter is capable of expressing a GOI in at least one (but not all) specific tissue of the complete promoter of Figure 8. Therefore with this particular aspect of the invention, the promoter having an inactivated portion can still function as a promoter (hence it is still called a promoter) but wherein the promoter is capable of

expressing a GOI in one or more, but not all, of the tissues where a GOI is expressed by the complete promoter shown in Figure 8.

Examples of partial inactivation include altering the folding pattern of the promoter sequence, or binding species to parts of the nucleotide sequence, so that a part of the nucleotide sequence is not recognised by, for example, RNA polymerase. Another, and preferable, way of partially inactivating the promoter is to truncate it to form fragments thereof. Another way would be to mutate at least a part of the sequence so that the RNA polymerase can not bind to that part or another part.

Accordingly, for a preferred embodiment of the present invention there is provided a promoter comprising a nucleotide sequence corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof but wherein the promoter is truncated. The term "truncated" includes shortened versions of the promoter shown in Figure 8.

Accordingly, for a preferred embodiment of the present invention there is also provided a promoter comprising a nucleotide sequence corresponding to the 5.5. Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof, but wherein the promoter does not contain at least the nucleotide sequence of any of one the sequences shown as Seq.I.D.No.s 4 – 16.

Furthermore, for a preferred embodiment of the present invention there is also provided a promoter comprising a nucleotide sequence corresponding to the 5.5. Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof, but wherein the promoter does not contain at least the nucleotide sequence shown as Seq.I.D. No. 1.

The term "construct" – which is synonymous with terms such as "conjugate", "cassette" and "hybrid" – includes a GOI directly or indirectly attached to the promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the *Sh1*-intron or the ADH intron,

intermediate the promoter and the GOI. The same is true for the term "fused" in relation to the present invention which includes direct or indirect attachment. In each case, it is highly preferred that the terms do not cover the natural combination of the wild type alpha amylase gene ordinarily associated with the wild type gene promoter and the wild type promoter and when they are both in their natural environment.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a plant cell into which it has been transferred. Various markers exist which may be used in, for example, plants – such as mannose. Other examples of markers include those that provide for antibiotic resistance – such as resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

The term "GOI" with reference to the present invention means any gene of interest. A GOI can be any nucleotide that is either foreign or natural to the organism (e.g. plant) in question.

Typical examples of a GOI include genes encoding for proteins and enzymes that modify metabolic and catabolic processes. For example, the GOI may be a protein giving added nutritional value to the plant as a food or crop. Typical examples include plant proteins that can inhibit the formation of anti–nutritive factors and plant proteins that have a more desirable amino acid composition (e.g. a higher lysine content than the non–transgenic plant).

The GOI may even code for an enzyme that can be used in food processing such as chymosin, thaumatin and alpha–galactosidase. The GOI may even code for an agent for introducing or increasing pathogen resistance. The GOI may even be an antisense construct for modifying the expression of natural transcripts present in the relevant tissues.

The GOI may even code for a non–natural plant compound that is of benefit to animals or humans. For example, the GOI could code for a pharmaceutically active protein or enzyme such as any one of the therapeutic compounds insulin, interferon,

human serum albumin, human growth factor and blood clotting factors. In this regard, the transformed cell or organism could prepare acceptable quantities of the desired compound which would be easily retrievable from, for example, the tubers.

Preferably the GOI is a gene encoding for any one of a protein having a high nutritional value, a pest toxin, an antisense transcript such as that for patatin, ADP-glucose pyrophosphorylase (e.g. see EP-A-455316), alpha-amylase (e.g. see EP-B-0470145), a protease antisense or a glucanase. A preferred GOI is an anti-sense sequence to the alpha-amylase gene described in EP-B-0470145.

The term 'organism' in relation to the present invention includes any organism that can activate the promoter of the present invention, such as amylase (e.g. alpha-amylase) producing organisms including plants, algae, fungi and bacteria, as well as cell lines thereof. Preferably the term means a plant or cell thereof, preferably a dicot, more preferably a potato.

The term 'transgenic organism' in relation to the present invention means an organism comprising either an expressable construct according to the present invention or a product of such a construct. For example the transgenic organism can comprise an exogenous nucleotide sequence (e.g. GOI as herein described) under the control of a promoter according to the present invention; or a native nucleotide sequence under the control of a partially inactivated (e.g. truncated) promoter according to the present invention.

The terms "cell", "tissue" and "organ" include cell, tissue and organ *per se* and when within an organism. For one class/type of promoters according to the present invention the term means potato tuber cell, tissue or organ and/or potato root cell, tissue or organ and/or potato sprout cell, tissue or organ and/or potato stem cell, tissue or organ. Preferably, the term means means just or at least a potato tuber cell, tissue or organ.

Preferably the expressable construct is incorporated in the genome of the organism. The term incorporated preferably covers stable incorporation into the genome.

The term 'nucleotide' in relation to the GOI includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA.

The term "expression vector" means a construct capable of *in vivo* or *in vitro* expression.

The term "transformation vector" means a construct capable of being transferred from one species to another - such as from an *E.Coli* plasmid to a plant cell.

Even though the promoters of the present invention are not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to put the present invention into practice.

Some of these background teachings are included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material. Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries a promoter or construct according to the present invention and which is capable of introducing the promoter or construct into the genome of a plant such as a plant of the family *Solanaceae*, in particular of the genus *Solanum*, especially *Solanum tuberosum*.

The vector system may comprise one vector, but comprises preferably two vectors; in the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), Binary Vectors, *Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above. A non-limiting example of such a Ti plasmid is pGV3850.

The promoter or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

As will be understood from the above explanation, the vector system of the present invention is preferably one which contains the sequences necessary to infect a plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct. Furthermore, the vector system is preferably an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

In the construction of a transgenic plant the promoter or construct may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example of a useful microorganism is *E. coli*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*, it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*.

The Ti-plasmid harbouring the promoter or construct of the invention is thus preferably transferred into a suitable *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the promoter or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists,* eds.: D.S. Ingrams and J.P. Helgeson, 203-208. See also Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27).

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 332, pUC series, M13 mp series, pACYC 184 etc. In such a way, the construct or promoter can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used a sequence analysis, a restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid. After each introduction method of the desired promoter or construct in the plants further DNA sequences may be necessary. If for example for the transformation, the Ti- or

Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells is being intensively studied and is well described in EP 120 516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblasserdam, 1985, Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46 and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is another simple technique which may be employed. Typically, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*, e.g. in a solution. Alternatively, the infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a root, a stem or another part of the plant. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc. Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

In summation therefore the present invention therefore relates to a promoter and, also to a construct comprising the same. In particular the present invention relates to the use of a promoter for the expression of a GOI in an cell/tissue/organism such as one or more specific tissues of a plant, in particular a dicot plant such as a potato.

More in particular, in a preferred embodiment, the present invention relates to a partially inactivated (such as truncated) type 3 α-amylase promoter.

The present invention also relates to the application of one class of partially inactivated gene promoters to express a GOI specifically in the tuber tissue of a dicot – especially a potato plant.

The following sample has been deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St Machar Drive, Aberdeen, Scotland, AB2 1RY, United Kingdom, on 26 August 1994:

DH5alpha-gPAmy 351 (Deposit No. NCIMB 40682).

This sample is an *E. Coli* bacterial stock containing the plasmid pBluescript (see Figure 7 for a general map thereof) containing an *EcoR*1 5.5 genomic DNA fragment isolated from potato (*Solanum tuberosum*). The *EcoR*1 5.5 fragment contains the promoter region and part of the 5' untranslated sequence of the structural gene of a potato alpha-amylase gene. The plasmid was formed by inserting the *EcoR*1 5.5 kb potato fragment into the polylinker of the vector pBS (Short et al [1988] Nuc. Acid. Res. 16:7583-7600). The promoter may be isolated from the plasmid by enzyme digestion with *EcoR*1 and then extracted by typical separation techniques (e.g. gels).

The following sample has been deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St Machar Drive, Aberdeen, Scotland, AB2 1RY, United Kingdom, on 20 October 1994:

DH5alpha-pJK4 (Deposit No. NCIMB 40691).

This sample is an *E. Coli* bacterial (DH5alpha-) stock containing plasmid pJK4 (described later).

The present invention will now be described only by way of examples in which reference is made to the following Figures:

Figure 1 shows a restriction enzyme map;

Figure 2 shows a restriction enzyme map;

Figure 3 shows a nucleotide sequence of a promoter according to the present invention;

Figure 4 is a pictorial representation of some deletions made to the sequence of Figure 2;

Figure 5 is a pictorial representation of some deletions made to the sequence of Figure 2;

Figure 6 shows a series of primer sequences;

Figure 7 shows a map of pBlueScript KS (2.96 kb) and a map of pBlueScript M13 (3.2 kb);

Figure 8 is a restriction enzyme map;

Figure 9 is a restriction map of pJK4; and

Figure 10 is a map of pEPL.

In more detail, Figure 1 is a restriction enzyme map of genomic clone gPAmy 351 isolated from the potato variety *Saturna*, in which the arrow indicates the position of the promoter, the closed bar indicates the position of coding sequences, H=*Hind*III, E=*EcoR*I, S=*Sal*I, ATG = initiation codon of the alpha-amylase coding sequence and A star marks the position of the 5.5 kb *EcoR*I fragment.

Figure 2 is a sequence map of the alpha-Amy 3 promoter in which the arrows show the extent of the sequence reactions, the position of the HE fragment is shown in B together with the 5' sequenced part of the promoter deletion series, the names of the individual fragments (see also Figure 4) are given above the arrows, ATG = initiation codon of the alpha-amylase coding sequence, and the deletion fragments chosen for functional analysis are indicated by asteriks.

Figure 3 is a nucleotide sequence of part of the alpha-Amy 3 promoter in which the restriction sites are bold faced, TATA, CCAAT and ATG sites are underlined, the position of the proposed CAP site and the untranslated leader sequence are indicated, and the 166 bp nucleotide sequence sandwiched between the two highlighting lines (i.e. from nucleotide position -857 to nucleotide position -691) is represented as SEQ. I.D. No. 1 (see later). This 166 bp nucleotide sequence may be refered to as the "delta" fragment or sequence.

Figures 4 and 5 represent two deletion series of the alpha-Amy 3 promoter with those of Figure 5 being used for functional analysis; Figure 6 shows a series of primer sequences for use with the present invention wherein Uni = T7 primer and Rev = T3 primer.

In further detail, the nucleotide sequence of Figure 3 is part of the promoter sequence of Figure 1 (discussed below) and part of the alpha-amylase structural gene, which in turn is part of the sequence of Figure 8. Part of this nucleotide sequence forms part of the sequences shown in the attached sequence listings. The nucleotide sequence of Figure 3 is repeated as Seq. I.D. No. 17.

Figure 8 is a pictorial representation of plasmid gPAmy351. The highlighted portion is a *EcoR*1 - *Sal*I fragment isolated from potato (*Solanum tuberosum*) - which is the same as the fragment shown in Figure 1. The *EcoR*1 - *Sal*I fragment contains the *EcoR*I 5.5 kb fragment (called subclone Eco 5.5) -which is indicated by the line shown at the bottom of the drawing. The *EcoR*I 5.5 kb fragment contains the promoter region and part of the 5' untranslated sequence of the structural gene of a potato α-amylase. The following restriction enzyme sites are shown in Figure 8: E: *EcoR*I, Ha: *Hae*III, Sp: *Ssp*I, H: *Hind*III, P: *Pvu*I, S: *Sal*I. In addition putative CAAT and TATA boxes and the ATG initiation site are shown. Introns are shown as open bars and exons as closed bars.

The *EcoR*I 5.5 fragment is cloned into a pBluescript M13-plasmid (shown in Figure 7) or a pBSK-plasmid (also shown in Figure 7).

For convenience, Chart 1 correlates the sequence references shown in the attached Figures with the sequences shown in the attached Sequence Listings.

CHART 1

SEQUENCE I.D. No.	FIGURE No./FIGURE REFERENCE
1	4/Delta
2	4/EH
3	4/8.5-E
4	4/9.5-7
5	4/8-17
6	4/7-1
7	4/6-15
8	4/6-13
9	4/6.5-4
10	4/5-24
11	4/4-1
12	4/4-2
13	4/1-8
14	4/1-6
15	EH8
16	-/-
17	3
2	5/HE
3	5/HFP8
7	5/HFP6
11	5/HFP4
15	5/EH8

In the following examples, the following materials and methods were used and followed, respectively.

MATERIALS AND METHODS

Plant material

Root tissue were harvested from flowering potato (*Solanum tuberosum*, cv. *Saturna*) plants. The roots were sliced directly into liquid nitrogen and 10–15g portions were stored at −80°C until use.

Bacterial strains

DH5α™ (BRL): F^-, endA1, hsdR17(r_k^-, m_k^+), supE44, thi-1, λ^-, recA1, gyrA96, relA1, (argF-lacZYa)U169, σ80dlacZ ΔM15

JM109(1): recA1, endA1, gyrA96, thi, usdR17, supE44, relA1, λ^-Δ (lac⁻proAB),[F', traD36, proAB, $LacI^qZ$ ΔMI5]

PLK17 (Stratagene): hsdR-M+, mcrA-,B-, lac-, supE, gal-

LE392 (2,3): supE44, supF58, hsdR514, galK2, galT22, metB1, trpR55, lac41

LBA4404: contains the disarmed pTiAch5 plasmid pAL4404 in the streptomycin resistant derivative of the *Agrobacterium tumefaciens* strain Ach5 (4).

Phages and plasmids

λ EMBL3:	see reference (5)
pBR327:	see reference (6)
pBS+, pBS-:	see reference (7)
pBSK+, pBSK-:	see reference (7)
pBI101, pBI121:	see reference (8,9)

Media and plates

L-Broth (LB) medium:

Per litre: 5g of yeast extract, 5g of NZ-amide, 5g of NaCl, 5g of bacto-peptone. Autoclave.

LB-plates:

LB medium plus 15g Bacto agar per litre. Autoclave. Pour into plastic petri dishes (25 ml/dish).

Amp-plates:

As LB-plates plus 35 mg ampicillin per litre after autoclaving.

AXI-plates:

As LB-plates plus 35 mg ampicillin, 120 mg IPTG (isopropylthiogalactoside), 40mg Xgal (dissolved in dimethylformamide) per litre after autoclaving.

Xgal: 5-bromo-4chloro-3indolyle-β-D-galactoside.

Kan-plates:

As LB-plates plus 50 mg kanamycin per litre after autoclaving.

YMB medium:

Per litre:0.66 g K2HPO4 - 3H2O. 0.2 g MgSO4. 0.1 g NaCl. 10.0 g Mannitol. 0.4 g Yeast extract. Adjust pH to 7.0. Autoclave.

Liquid MBa medium:

Per litre:4.4 g MS salts (Murashige and Skoog basal salt (10),Sigma). 20 g sucrose. pH is adjusted to 5.7 with NaOH.

Solid MBa medium:

As liquid MBa medium plus 0.8 % Difco agar.

MBa co:

As solid MBa medium plus 0.5 mg t-Zeatin (trans-isomer, Sigma) and 2.0 mg 2,4 D (2,4-dichlorophenoxacetic acid, Sigma) per litre.

Solid MBb medium:

As solid MBa medium but instead of 20 g sucrose 30 g sucrose is added per litre.

Water:

The water used in Materials and Methods was always distilled and autoclaved before use.

<u>Isolation of high MW genomic potato DNA</u>

In order to gain high molecular weight genomic DNA a procedure essentially as described by Fischer and Goldberg (11), was followed. This include first isolation of nuclei followed by preparation of the nuclear DNA.

10-15g *Saturna* root tissue were ground to a fine powder in liquid nitrogen and homogenized in 100 ml H buffer (1xH buffer(1l): 100ml 10xHB, 250ml 2M sucrose, 10 ml 100mM PMSF, 1ml β-mercaptoethanol, 5 ml Triton X-100, 634ml H_2O. Adjust to pH 9.5. Add β-mercaptoethenol just before use. 10xHB: 40mM spermidine, 10mM spermine, 0.1mM Na-EDTA, 0.1mM Tris, 0.8mM KCl, adjusted to pH 9.4-9.5 with 10N NaOH. PMSF: phenylmethylsulfonyl fluoride dissolved in ethanol). The resuspended plant material was filtered through a 70μm nylon filter (Nitex filter, prewetted in 1xH buffer). The resulting filtrate was poured into two centrifuge bottles

(Sorvall GSA) and the nuclei were pelleted at 4000 r.p.m for 20 min at 4°C. The supernatant was discarded and the pellets were gently resuspended by adding 20 ml 1xH buffer per tube and then swirling the tubes carefully. The nuclei were pelleted again at 4000 r.p.m. for 20 min at 4°C, the supernatant removed and the pellets resuspended gently in 10 ml 1xH buffer. The supernatant were pooled and 20 ml cold lysis buffer (lysis buffer: 2% Sarcosyl, 0.1M Tris, 0.04M Na_2-EDTA) was added dropwise while the solution was stirred gently. Immediately after the last drop of lysis buffer was added, 0.972g CsCl/ml solution was stirred gently into the solution (the solution should now be at room temperature). The resulting solution was centrifuged for 45 min at 10 krpm, 4°C. The supernatants were carefully removed using a pasteur pipet avoiding any protein debris floating on the top or disturbing the pellets. The volume of the supernatants were determined and 0.2 mg ethidium bromide/ml was added. The DNA solution was gently poured into quickseal polyallomer tubes, which were then sealed. The tubes were centrifuged in a Beckman VTI 65 rotor at 18°C and 40 k r.p.m. for 38 h.The genomic band was removed under UV-light with a 15-18 gauge needle attached to a 5-ml syringe and poured gently into a 5 ml polyallomer tube.

The tube(s) was then filled with a 1.57 g/ml CsCl solution in 50mM Tris-Hcl(pH 9.5), 20 mM Na_2-EDTA. 75 μl ethidium bromide (5mg/ml) was added/tube. The tubes were centrifuged in the VTI 65 rotor at 18°C and 46 k r.p.m. for 17 h. The genomic band was removed under long-wave UV light and the ethidium bromide was extracted with CsCl-saturated isopropanol (7 to 8 times).

The CsCl was removed from the DNA by dialysis in TE-buffer (1xTE: 10 mM Tris-HCl, 1 mM Na_2-EDTA pH 8.0) at 4°C for 18 h with three changes.The high MW genomic potato DNA was not further precipitated and was kept at 4°C.

Construction of a potato genomic library

High MW genomic potato DNA was prepared from cv *Saturna* roots as decribed above. The quality of the DNA was tested by restriction enzyme digestion and gel electrophoresis.

The genomic DNA was partially digested with Sau3A and the created fragments (9-23 kb) were inserted into the BamHI site of the λ EMBL3 vector(4). Approximately $1.1x10^6$ independent isolates were plated and amplified to form a permanent library (12). Plaque hybridization was used to screen the library for α-amylase genes.

Screening of the library

Screening of the potato genomic library was carried out essentially as described by references 13 and 14. The pfu/ml (pfu:plaque forming unit) of the amplified genomic library was determined in duplicate prior to the screening. Infection competent cells (PKL17 or LE392) were prepared by inoculating the cells in 30 ml fresh L-Broth containing 0.2% sucrose and 10 mM $CaCl_2$. The cells were cultivated for 4-5 h at 37°C before 0.1 vol of cold $CaCl_2$ was added and kept on ice until use. 100 µl phages diluted in phagebuffer to give an appropriate number of pfu (1xphagebuffer: 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 20 mM NaCl) were mixed with 25-100 µl freshly made cells (dependent on the actual number of cells) and incubated at 37°C for 15-20 min. The suspension was mixed with 3 ml warm (42°C) 0.8-1% top agar containing 10 mM $MgCl_2$ and plated out on dry LB plates.

LB plates of 22x22cm (dried for 3-4 h at 37°C) were used for screening of the genomic library. Each plate contained approximately $2x10^5$ plaques, which were mixed with 1 ml of infection competent cells (prepared as above) and incubated for 20 min at 37°C. This mixture was then added to a 25 ml of warm (42-45°C) 0.3% top agarose with 10 mM $MgCl_2$ and the solution was poured onto a fresh dry LB plate. The large LB plates were incubated (not upside down) overnight at 37°C. Phages from the plaques were transferred to Hybond N filters (Amersham) in duplicates. The plates

were placed at 4°C for 1 to 2 h to prevent the agarose layer from sticking to the filters.

The plates were placed on ice, just before use and they remained on the ice when working with the filters.Two Hybond N filters and a plate were marked for orientation of the filters . The first filter was laid on the plaques for 45 sec; then floated on denaturation buffer (0.5 M NaOH, 1.5 M NaCl) for 7 min, with the phages facing up, then floated on neutralization buffer (0.5 M Tris-HCl (pH 7.4), 3 M NaCl) for 2 times 3 min and finally washed in 2xSSC (1xSSC: 0.15 M NaCl, 0.015 Na-citrate). The filter was air dried and the phage DNA was fixed to the membrane by UV crosslinking. The second filter was laid on the same plate, after the first, for 120 sec and then treated as the first. These filters were used in plaque hybridization following the Hybond N membrane protocol according to suppliers (Amersham) instructions. X-ray film from both the first and second Hybond N membrane was orientated so that the signals from both filters fitted each other.

The positive plaques were cut with a scalpel (1x1 cm blocks) and submerged in 1 ml phagebuffer. The phage containing tubes were stored airtight (parafilm) at 4°C after 2-3 drops of chloroform has been added. The plaque containing plates (22x22 cm) were stored by placing a piece of soaked (chloroform) filterpaper in the lid. The plates were also stored airtight at 4°C with the plaques facing up. Further purification of the positive plaques were done by plating dilutions of the stock tube (containing the 1x1 cm block) with freshly prepared cells and plate them on round LB plates with 1% warm (42°C) top-agar and 10 mM $MgCl_2$.

New filter prints were made with Hybond N following the procedure outlined above with the 22x22 cm plates. Plaques which gave positive signal were isolated by sticking the tip of a pasteur pipette though the plate and transfer it to 500 μl phage buffer.

A new series of dilutions were made, plated and the respective filters hybridized until the positive plaques were purified.

The phages were stored airtight, at 4°C either in the 500 µl phagebuffer with 1 drop of chloroform, or as phages isolated from a plate lysate. The plate lysate stock was made as decribed by (14).

Isolation of recombinant λ DNA

Large-scale preparations followed the method decribed in (14), which include banding the recombinant phage DNA on a CsCl gradient. Two versions (A,B) of a small-scale preparation were used as follows:

A) LE392 cells were inoculated in LB with 0.2% maltose and 10 mM $MgCl_2$ and grown O/N at 37°C. The cells were pelleted by centrifugation for 10 min, at 4°C in a Sorvall centrifuge, and resuspended gently in 1 volume of cold 10 mM $MgSO_4$. The cells were stored at 4°C until use. Five single plaques from a plate were transferred to 500 µl phagebuffer and allowed to stand for 2-2½ h at 4°C. After votex of the tube 100 µl of the liberated phages were mixed with 200 µl freshly prepared LE392 cells. Alternatively 50-100 µl liberated phages from aplate lysate were mixed with the cells. Phages and cells were incubated for 20 min at 37°C and then added to a prewarmed (37°C) 25 ml LB with 20 mM $MgSO_4$ and 30 mM Tris-HCl pH 7.5 and incubated, shaking O/N at 37°C. A further 10 ml prewarmed LB with 20 mM $MgCl_2$ and 30 mM Tris-HCl pH 7.5 was added and the mix incubated for 1-2 h shaking at 37°C. After clear lysis (eventuel a few drops of chloroform was added to help) and the solution was centrifuged at 8000 r.p.m. for 10 min at 4°C. The supernatant was transferred to a new tube and centrifuged again if necessary to remove cell debris.

The recombinant λ DNA was then purified using a Qiagen column following the suppliers instructions (15).

B) The procedure was as under A) until after the first centrifugation of the O/N culture. The supernatant was transferred to a new tube and DNase was added corresponding to 1 µg/ml. The solution was incubated 30 min at 37°C and then 1 volume of cold 20% PEG, 2 M NaCl mixed in phagebuffer was added and the

mixture was incubated 1 h on ice. The phages were pelleted by centrifugation for 20 min, 4°C at 10 krpm. The PEG pellet was resuspended in 400 µl phagebuffer and transferred to an eppendorf tube. 1 µl of RNase (10 mg/ml) is added and the tube incubated for 30 min at 37°C. Then 8 µl 0.25 M Na_2-EDTA, pH 8.0 and 4 µl 10% SDS were added, the tube was incubated a further 15 min at 68°C. The mixture was allowed to gain room temperature and then an equal phenol saturated with TE-buffer (1xTE: 10 mM Tris pH 7.5, 1 mM Na_2-EDTA) was used to extract the DNA. A equal mixture of saturated phenol-chloroform was used to extract the upper aqueous phase and a final chloroform extraction was done. The upper phase was transferred to a new tube and the solution was made 0.3 M Na-acetate and 2-3 vol cold ethanol was added. The precipitation of the DNA was accomplished by storing at O/N at -20°C, centrifuging for 5 min and resuspend the pellet in 50-100 µl TE-buffer. The amount and quality of the recombinant phage DNA was tested by restrictions enzyme digest and agarose (0.8-1%) gel electrophoresis (16).

Preparation of plasmid DNA

The plasmid preparation was as described in EP-B-0470145. In particular, small scale preparation of plasmid DNA was performed as follows. Bacterial strains harbouring the plasmids were grown overnight in 2 ml L-Broth (LB) medium with ampicillin added (35 µg/ml).

The operations were performed in 1.5 ml Eppendorf tubes and centrifugation was carried out in an Eppendorf centrifuge at 4°C. The cells from the overnight culture were harvested by centrifugation for 2 min., washed with 1 ml 10 mM Tris-HCl (pH 8.5), 50 mM EDTA and centrifuged for 2 min. The pellet was suspended in 150 µl of 15% sucrose, 50 mM Tris-HCl (pH 8.5), 50 mM EDTA by vortexing. 50 µl of 4 mg/ml lysozyme was added and the mixture was incubated for 30 min. at room temperature and 30 min. on ice. 400 µl ice cold H2O was added and the mixture was kept on ice for 5 min, incubated at 70-72°C for 15 min. and centrifuged for 15 min. To the supernatant was added 75 µl 5.0 M Na-perchlorate and 200 µl isopropanol (the isopropanol was stored at room temperature), and the mixture was centrifuged for 15

min. at 4°C. The pellet was suspended in 300 µl 0.3 M Na-acetate and 2-3 vol. cold ethanol was added. Precipitation was accomplished by storing at either 5 min. at -80°C or O/N at -20°C, centrifuging for 5 min., drying by vacuum for 2 min. and redissolving the pellet in 20 µl H_2O. The yield was 5-10 µg plasmid DNA.

Large scale preparation of plasmid DNA was accomplished by simply scaling up the small scale preparation ten times. Working in 15 ml corex tubes, all the ingredients were scaled up ten times. The centrifugation was carried out in a Sorvall cooling centrifuge at 4°C. Only changes from the above will be mentioned in the following. After incubation at 70-72°C, the centrifugation was for 30 min. at 17,000 rpm. After adding isopropanol and after adding cold ethanol, the centrifugation was for 15 min. at 17.000 rpm. The final plasmid DNA pellet was suspended in H_2O and transferred to an Eppendorf tube and then given a short spin to remove debris. The supernatant was adjusted to 0.3 M Na-acetate and 2-3 vol. cold ethanol were added. The pellet was resuspended in 40 µl H_2O. The yield was usually 20-28 µg plasmid DNA.

To obtain very pure plasmid DNA, 200-300 µg of isolated plasmid DNA from the upscaled method were banded on a CsCl gradient. Solid CsCl was mixed with H_2O (1:1 w/v) and 0.2 mg/ml ethidium bromide was added. The solution was poured into a quick-seal polyallomer tube and the plasmid DNA, mixed with solid CsCl (1:1 w/v). The tube was filled, sealed and centrifuged in a Beckman VTI 65 rotor at 15°C, 48.000 rpm for 16-18 hours. The centrifuge was stopped by without using the brake. The banded plasmid DNA was withdrawn from the tubes using a syringe and the ethidium bromide was extracted with CsCl-saturated isopropanol 7-8 times. The CsCl was removed by dialysis in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA for 48 hours with three changes of buffer. The DNA was precipitated by adjusting to 0.3 M Na-acetate and adding 2-3 vol.cold ethanol.

The small scale plasmid preparation from *E. coli* was usually followed by a LiCl precipitation to remove RNA from the DNA solution. The small scale prepared plasmid DNA was dissolved in 100 µl destilled water. 1 vol of 5M LiCl was added and the mixture incubated at -20°C for 30 min followed by centrifugation at 12,000

rpm. for 15 min, 4°C. The supernatant was transferred to a new eppendorf tube and 2 vol TE buffer or water was added. Precipitation with 2.5 vol of 96% ethanol was accomplished by storing either 10 min. at -80°C, or O/N at -20°C. The DNA was precipitated by centrifuging for 15 min. 12,000 rpm ,at 4°C, drying by vacuum for 2 min and redissolving in 18 μl of TE or water.

Restriction enzyme digestion

The protocol followed was that outlined in EP-B-0470145. In particular, all restriction endonucleases were from Biolabs , Amersham or Boehringer Mannheim and were used according to the supplier's instructions. 1 unit of enzyme was used to 1 μg of DNA and incubation was for 2 hours.

The buffer was changed in double digestions, by changing the volume or by adding the necessary ingredient according to the enzyme instructions.

Labelling of DNA

A random primed DNA labelling kit (Boehringer Mannheim) was used according to the suppliers instructions. Briefly, 2 μl DNA fragment (25-50 ng) is mixed with 8 μl H_2O and incubated at 95°C for 10 min to denature the DNA. Spin shortly and place on ice. Then add 1 μl dGTP, dATP and dTTP of each, 2 μl reactionsmix and 5 μl (approx. 50 μCi $dCTP^{32}$). 1 μl Klenow enzyme starts the reaction and the tube is incubated at 37°C for 30 min. Then place on ice. The labelled DNA fragment was purified using an ELUTIP column (Schleicher & Schuell). The column was prepared by prerunning (gravity) it with 3 ml high salt buffer (1.0 M NaCl, 20 mM Tris-HCl (pH 7,5), 1.0 mM EDTA), followed by 5 ml low salt buffer (0.2 M NaCl, 20 mM Tris-HCl (pH 7,5), 1.0 mM EDTA). 250 μl low salt was added to the labelling tube and the entire solution was laid on the prepared column. Then the column was washed with 2x400 μl low salt followed by 3x200 μl high salt. The eluted radioactive probe was then heat denatured and used in hybridization.

Southern transfer and Hybridization

The DNA fragments to be transferred were fractionated on non-denaturing agarose gels (14) and transferred to either Hybond™ N or Hybond™ N+, positively charged nylon membrane (Amersham Life Science) by Southern blotting (17,18). Hybridization to the Hybond™ N nylon membranes followed the supplier instructions (18).

Preparation of vectors

The preparation of vectors was as decribed in EP-B-0470145 as follows: Vectors (pBS-/+ or pBSK-/+) were digested with one or two restriction enzymes, extracted twice with saturated phenol (the phenol was first mixed with 0.1 M Tris-HCl, then mixed twice with TE-buffer (10 mM Tris-HCl, pH 8, 1 mM Na_2-EDTA)) and once with chloroform and precipitated with 0.3 M Na-acetate and 2.5 vol cold ethanol. The pellet was rinsed in 70% cold ethanol and dissolved in H_2O, giving a concentration of 25-50 ng/μl. The vectors were tested for background before use (self-ligation with and without T4-DNA-ligase). If necessary the vector was treated with Alkaline phosphatase (Boehringer Mannheim) as described by the supplier. After such a treatment the resulting pellet was resuspended in H_2O to give a final concentration of 10 ng/μl.

Ligation

The phage DNA or plasmid comprising a fragment to be subcloned was digested with one or more restriction enzymes and run in either a 5% acrylamide gel or an appropriate agarose gel. The fragment to be subcloned was isolated from the gel either by electroelution as decribed in (14) or using a GENECLEAN II Kit (BIO 101 Inc., La Jolla, California) following the suppliers instructions.

Various ratios of fragment to vector were used (from 2:1 to 5:1, based on the number of molecules) in the ligation reaction. 1 μl (10-100 ng) of a solution containing the vector was combined with the fragment, 1 μl of T4-ligation buffer (10x(20 mM

Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 0.6 mM ATP, 10 mM dithiotheitol)) and 1 μl of T4-DNA ligase (Boehringer Mannheim) were added to a mixture of fragment and vector to a total volume of 10 μl. The reaction was incubated at 15°C O/N if the ligated DNA fragments had sticky ends.

If the DNA had blunt ends, the incubation was at room temperature for 1 hour. The ligation mixture was stored at -20°C if not used immediately, usually 5 μl of the ligation mix was used for transformation.

DNA fragments treated with a DNA blunting kit (see "subcloning and sequencing") were ligated following the DNA blunting kit's protocol (Amersham).

Preparation of competent *E. coli* cells and transformation

This was done according to the protocols laid down in EP-B-0470145 as follows:

JM109 cells (or DH5α) were inoculated in 4 ml L-Broth made to 10 mM $MgSO_4$ and 10 mM $MgCl_2$. The ccells were grown O/N at 37°C. 1 ml of the O/N culture was added to 40 ml prewarmed (37°C) LB medium (with 10 mM $MgSO_4$ and 10 mM $MgCl_2$). The culture was shaken at 250-275 rpm for 1 to 2 h until the OD_{450} reached 0.8-0.9. The cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The pellet was gently resuspended in 30 ml of cold 0.1 M $CaCl_2$, another centrifugation pelleted the cells again and they were then resuspended in 15 ml of cold 0.1 M $CaCl_2$. The suspension was placed on ice for 20 min followed by a centrifugation as before. Finally, the cells were gently resuspended in 3 ml of cold 0.1 M $CaCl_2$ and placed on ice for at least 1 h before they were ready to use for transformation (19).

5 μl of ligation mix was combined with 95 μl of cold sterile TCM (10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$, and 0.2 ml of the competent cells. The mixture was allowed to stand for at least 40 min on ice, then 5 min at 37°C (or 2 min at 42°C). The solution was transferred to 0.8 ml of L-Broth, 10 mM MgSO4, 10 mM

$MgCl_2$, and incubated for 45 min at 37°C and then plated out on 5 AXI or other plates (as e.g. Amp-plates) at 0.2 ml/plate.

The plates were allowed to stand 10 min before being inverted and incubated O/N at 37°C. They were stored in plastic bags upside down at 4°C.

ExoIII/Mung Bean Nuclease Deletions

A deletion series of a subcloned larger genomic fragment was performed using a ExoIII/Mung Bean Deletion Kit (Stratagene). The subclone selected for the deletion series was purified by banding twice on a CsCl gradient (see "Preparation of plasmid DNA") to obtain high amounts of supercoiled plasmid DNA. Generation of the deletions was performed using the ExoIII/Mung Bean Deletion kit following the suppliers instructions. The temperature during the ExoIII treatment was 23°C since at that temperature approximately 125 bp should be removed per min.

Purification of primers following synthesis on a DNA Synthesizer

The primer was synthesized on a polystyrene support column (Applied Biosystems, 393 DNA/RNA Synthesizer) and was eluted from the column with NH_4OH. The column was broken open and 1.5 ml NH_4OH was added to the polystyrene material in a small glass tube. The mixture was incubated at 85°C for 1 hour followed by 5 min on ice. The supernatant containing the single stranded DNA was transferred to eppendorf tubes, and the NH_4OH was evaporated in a vacuum centrifuge for at least 3 h.Pellet was resuspended in 200 μl destilled water and precipitated with 550 μl ethanol and 20 μl sodium acetate. The pellet was resuspended in 200 μl water and precipitation with ethanol and sodium acetate was repeated. Finally the pellet was resuspended in 100 - 200 μl destilled water and the OD_{260} was measured by a Gene Quant RNA/DNA calculator (Pharmacia) of single stranded DNA is calculated. An OD_{260} of 1 corresponds approx. to 33 μg/ml single stranded DNA.

Subcloning and sequencing

Purified λ DNA was digested with appropiate restrictions enzymes and the generated fragments were isolated from agarose gels using a GeneClean Kit (BIO 101 Inc., La Jolla, California) according to the suppliers instructions.

Genomic DNA fragments (or fragments obtained from plasmids) were ligated into the polylinker region of the BlueScribe vector pBS-/+ (or pBSK-/+, Stratagene). After transforming an *E. coli* strain with the ligated plasmid the recombinant subclones could be selected by plating on AXI plates (they will be white and the nonrecombinant clones will be blue when the vector is a pBlueScript plasmid,(107)).

Plasmid DNA from putative subclones were digested with appropiate restriction enzymes, subjected to gelelctrophoresis and after Soutern blotting, hybridized with an appropiate labelled DNA probe, to verify the origin of the inserted fragment.

The generated pBS genomic DNA subclones were then sequenced according to the plasmid preparation protocol outlined in EP-B-0470145. In this regard, the plasmid (double stranded template) to be sequenced was purified by the plasmid small scale preparation method. The DNA was denatured in 0.2 M NaOH (5 min at room temperature) the mixture was neutralised by adding 0.4 vol of 5 M ammonium acetate (pH 7.5) and then precipitated with 4 vol. of cold ethanol (5 min at -80°C). The pellet was rinsed with 70°C cold ethanol and resuspended in 10 μl H_2O.

For subcloning of DNA fragments generated by using an ExoIII/Mung Bean Nuclease kit,the fragments were either blunted first or digested with a restriction enzyme, following by blunting.

The blunting of the DNA with an unknown end structure (after the ExoIII/Mung Bean treatment) or with cohesive ends was accomplished by using a DNA blunting kit (Amersham) following the suppliers instructions. The generated ligated (see "Ligation") deletions plasmids were transformed into DH5α competent cells and white

colonies, selected on AXI plates, were analysed for their insert by restriction enzyme digestion and further, by sequencing.

Sequencing was accomplished with a Sequenase™ DNA Sequencing Kit from United States Biochemical Corp., following the sequencing Protocol enclosed in the kit (Sequenase™ :Step by Step Protocols for DNA sequencing with Sequenase, 3rd Edition, United States Biochemical Corporation PO Box 22400 Cleveland Ohio 44122). The following modifications were however made to the suggested protocol. Instead of adding DTT, Labelling mix and ^{35}SdATP to the annealed DNA mix, 4 ml of ^{35}Sequetide (DuPont) was added.

In addition to T3 and T7 primers (Stratagene) a whole range of other primers generated on a DNA synthesizer (Applied Biosystems, 392 DNA/RNA Synthesizer) were used. 0.5 pmol of primer was used to sequence 1 pmol of plasmid.

The primer sequences are shown in the Figure 6.

The sequencing reaction were subjected to electrophoresis on 6 % or 8 % denaturing polyacrylamide gels for 1 to 4 hours at 40 W, then dried by a gel drier and autoradiographed for 3–48 hours at room temperature.

The denaturing sequencing gels were made from pre-mixed polyacrylamide solutions , Gel-Mix 6 and Gel-Mix 8 (GIBCO BRL, Life technologies, Inc) according to the manufacturers instructions.

Preparation of competent *Agrobacterium* cells and transformation

The LBA 4404 strain was kept at YMB plates (pH 7.0) containing 100 mg/ml of rifampicin (Sigma) and 500 mg/ml of streptomycin (Sigma). 2.5 ml of LB medium (pH 7.4) was inoculated with the bacteria. The suspension was left growing for 24 hours at 28°C in an incubation shaker at 300–340 rpm. The suspension was then diluted 1:9 with LB and incubated for another 2–3 hours at 28°C and 300–340 rpm.

When OD was 0.5–1, 25 ml aliquots of the cells were harvested in 50 ml tubes in a cooling centrifuge at 10.000 rpm, 5 min, 4°C. The tubes were placed on ice and the pellet resuspended in 0.5 ml of 20 mM $CaCl_2$. 0.1 ml aliquots of the resuspended cells were quickly frozen in 1 ml cryotubes in liquid nitrogen and stored at –80°C.

Transformation was accomplished using the freeze–thaw method (20) as follows:

A 0.1 ml aliquot of $CaCl_2$ competent LBA 4404 cells was thawed on ice and added 1 μg of plasmid DNA. The mixture was incubated at 37°C for 5 min. and added 1 ml LB (pH 7.4). Incubation at room temperature with shaking (100 rpm.) for 4 hours was followed by a quick spin at 10.000 rpm, 4°C for 30 sec. The pellet was resuspended in 100 μl LB and plated on a YMB plates containing 50 mg/l of kanamycin (Sigma).

The plates were incubated for 48 hours at 28°C or until the colonies had a suitable size.

This was the first round of selection. Only bacteria transformed with a plasmid containing the NPT II gene conferring kanamycin resistance is able to survive on the kanamycin plate.

For the second round of selection six of the obtained colonies were transferred to a YMB plate containing 100 mg/l of rifampicin, 500 mg/l of streptomycin and 50 mg/l of kanamycin. LBA 4404 is resistant to rifampicin and streptomycin and the plasmid confers resistance to kanamycin. The plates were incubated at 28°C until the colonies reached a suitable size (approx. 4–5 days).

The colonies were tested for their plasmid content. Plasmid preparations of the colonies were generated and the DNA was digested with appropriate restriction enzymes and run on a 1 % agarose gel to ensure that the plasmid and the inserted fragment had the right size. The digested DNA was blotted onto a Hybond N+ membrane and hybridised with an appropriate radioactively labelled probe (a fragment of the plasmid DNA or insert).

Storage of the transformed LBA 4404 was at -80°C. 2 ml LB medium containing 100 mg/l of rifampicin, 500 mg/l of streptomycin and 50 mg/l of kanamycin was inoculated with bacteria and incubated at 28°C for 48 hours with shaking (300-340 rpm). The suspension was diluted 1:1 with sterile 35 % glycerol and aliquoted into cryotubes, 800 µl per tube and stored at -80°C.

Transformation of potato

A culture of the transformed LBA 4404 bacteria were made by inoculating 2 ml of YMB (pH 7.0) with the bacteria and incubating at 28°C for 24 hours. The suspension was diluted 1:10 and incubated for another 18 hours. The bacteria was centrifuged at 10.000 rph, 4°C for 10 min. and the pellet rinsed twice with 2.5 ml of 2 mM magnesium sulfate, before resuspension in liquid MBa to an OD660 nm of 0.5.

The potato plant material used for transformation was maintained in vitro at MBa medium added 2 µM STS (21,22). By multiplication top shoots as well as nodes were applied, if the leaves were big they were removed. 5 shoots per container with 80 ml medium was left growing at 25°C and 30-35 days after subcultivation the nodes could be used for transformation.

The stems of micropropagated plants were cut just above and beneath the node so that only the internodes are used, these may possibly be divided so that the explants are approx. 4 mm long. The explants were floated in the bacterial suspension for 30 min. and blotted dry on a filter paper and transferred to co-cultivation plates (MBa co). The explants were covered with filter paper moistened in liquid MBa, and the plates were covered with cloth for 3 days and left at 25°C. The explants were then washed in liquid MBa containing 800 mg/l. 2 explants per ml were shaken for 18 hours, then blotted dry and transferred to selection medium.

The selection medium was solid MBb added 50 mg kanamycin, 800 mg carbenicillin (Duchefa), 0.1 mg GA_3 (Gibberellic Acid,Sigma) and 1 mg t-Zeatin per litre. The carbenicillin was added to kill any remaining *Agrobacteria*.

The explants were subcultivated every 3 weeks.

Regeneration of whole potato plants

Shoots from the explants which by subcultivation was more than 1 cm were harvested and transferred to a solid MBa medium containing 400 mg/l of carbenicillin, 2μM STS and 0.5 mg/l t-Zeatin. After approx.2 weeks the shoots were transferred to root-formation medium, that is solid MBa with 2 μM STS added. A 5 μM stock of STS was made from 0.19 g of $Na_2S_2O_3-5H_2O$ and 10.19 mg of $AgNO_3$ dissolved in 7 ml of water and sterilised by filtration.

After approx. 2 weeks the shoots had rooted and were ready for planting in soil.

The plantlets were rinsed in lukewarm water to remove residues of media and planted in small pots with TKS 2 instant sphagnum (Flora Gard, Germany). The plantlets were kept moist during the planting and watered after. The pots were placed in a "tent" of plastic with 100 % humidity and 21-23°C, until the plantlets were rooted in the soil. Then the tent was removed and the plants watered regularly.

After 4 weeks of growth the plants were potted into large pots (diameter 27 cm) and transferred to a growth chamber with 16 hours day 22°C and 8 hour night 15°C. When the plants had wiltered down, the tubers were harvested.

Generation of microtubers from *in vitro* propagated potato plants.

From an in vitro propagated plant or a selected shoot a node was cut of 5 mm under and 2 mm over the node. The leaf was removed and the explant was placed on a solid growth medium. The medium contained per litre: 4.4 g Murashige and Skoog (MS,(10)) basal salts (Sigma), 60 g sucrose, 2 mg BAP (6-Benzyl-aminopurine, Sigma), 2 g Gelrite (Scott Laboratories, Inc., Carson, California). The explants were incubated for 7 days at 20°C, with 16 h light/ 8 h dark. The plates were then wrapped in aluminium foil and kept in the dark at 20°C for 21-28 days. Then microtubers

could be harvested, one per explant.

Sprouts from microtubers were generated from the microtubers by cutting the tubers in two halves (from top to bottom). They were then placed on solid MBa medium and incubated in the dark for 7 days at 25°C and the newly developed sprouts could be GUS analysed.

Histochemical localisation of beta-glucuronidase (GUS) activity

The tissue was cut in small sections with a razor blade and placed in X-gluc (X-gluc: 5-bromo-4-chloro-3-indolyl- ß-glucoronide) is a solution of 50 mg X-gluc dissolved in a buffer with: 0.1 M $NaPO_4$ (pH 7.0), 1 mM $K_3(Fe(CN)6)$, 0.1 mM $K_4(Fe(CN)6)-3H_2O$, 10 mM Na_2EDTA and 3 % sucrose (23)) solution to cover the section.

Micro tubers were halved, pot grown tubers were sliced into thin slices, leaves were cut into pieces approx. 0.5 cm^2 and stem tissue was cut into slices approx. 1 mm thick.

The sections were incubated in X-gluc for 2-12 hours at 37°C. Care was taken to prevent evaporation. The X-gluc was removed and 96 % ethanol was added to the tissue sections to extract chlorophyll and other pigments. Incubation in ethanol was overnight at 5°C and the following day the tissue was transferred to a 2 % sucrose solution and after approx. 1 hour examined in a dissection scope.

Isolation of α-amylase genomic clones

Several cDNA clones encoding α-amylase from potato (*Solanum tuberosum*) had previously been isolated (described in EP-B-0470145). A *PstI-SalI* fragment from the plasmid pAmyZ3 (EP-B-0470145) encoding a partial α-amylase was used as probe (see "DNA labelling" in Materials and Methods) to screen the genomic potato λ DNA library (see "Construction of a potato genomic library" in Materials and

Methods). Screening of approx. $1.6x10^6$ phages was carried out as described in Materials and Methods. Two positive clones were isolated, gPAmy351 and gPAmy331 by three rounds of plaque purifications.

One clone (gPAmy331) was found to be unstable during isolation of the λ DNA (see the method in Materials and Methods), then only the gPAmy351 clone was analyzed in details.

A restriction enzyme map of the insert (insert size approx. 22kb) is shown in figure 1.

Mapping of the α-amylase encoding part of the genomic sequence was done by Southern transfer of various digests of the clone, followed by hybridization with the *PstI-SalI* fragment of pAmyZ3. gPAmy351 contain the whole promoter region of the α-amylase gene and in addition 1270 bp of the structural gene. This covers the sequences encoding 142 amino acids corresponding to approx. 1/3 of the total amino acid sequence encoded by the Amy3/4 type potato α-amylase (407 amino acids, see EP-B-0470145).

<u>Subcloning of the genomic fragment containing the α-amylase promoter</u>

The *EcoR*1 fragment of approximately 5.5 kb, indicated by an asterisk in figure 1, was subcloned from the gPAmy351 genomic clone into a dephosphorylated *EcoRI* site of a pBS-vector (see Materials and Methods).

This subclone was named Eco 5.5 and it contains the ATG initiation codon and sequences upstream of it (see the next paragraph for a more detailed description of the sequence). These upstream sequences will in the following be referred to as the α-Amy 3 promoter.

A large scale plasmid preparation of the Eco 5.5 plasmid was digested with *EcoRI* and *HaeIII*, this creates a 1350 bp fragment which includes the ATG initiation codon as

well as putative CAAT and TATA boxes.

As shown by others (eg see 24-31) it is often the sequence region counting from the ATG initiation codon and approx 1000-1500 bp upstream that includes the entire promoter, enough to mediate transcription of the gene at the right time and place. The *EcoRI-HaeIII* fragment was subcloned into a pBSK-vector, digested with *EcoRI* and SmaI and dephosphorylated by Alkaline phosphatase (see Materials and Methods). This subclone was named EH8 and the genomic potato fragment it carries was chosen for functional analysis. The identity of the insert in the EH8 plasmid was verified by sequencing with T3 and T7 primers (see Figure 2B and Materials and Methods).

Sequencing of the α-amylase promoter

Approximately 2900 bp of the insert in gPAmy351 (Figure 1) was sequenced by subcloning various fragments and using the primers shown in figure 6 (see Materials and Methods). This covers 1734 bp upstream of the initiation (ATG) codon and 1440 downstream. The sequence map of the region upstream of the ATG initiation codon is shown in figure 2A and the DNA sequence is shown in figure 3. This sequence is located near the 3' end of the gPAmy351 insert (see figure 1) covering part of the Eco 5.5 kb fragment and the *HindIII* and *EcoRI* sites upstream of the initiation codon, and thereby including the *HaeIII-EcoRI* (EH) fragment chosen for the functional analysis.

The α-Amy 3 promoter sequences from the ATG (A in position +1) codon and upstream to position -1734 (see figure 3) were compared with the (EMBL) database of published plant sequences (using the PC-gene program from IntelliGenetics, Inc.,California) and also compared with sequences of all organisms. There were no sequences with significant overall homology to the α-Amy 3 promoter. A TATA-box is located at position -365.

Comparing the α-Amy3 promoter with published DNA binding sites suggested a CAP site to be localized 21 bp downstream of the TATA box (position -344) and two

CAAT boxes, one at position -468, which is 103 bp upstream of the TATA box, and 124 bp upstream of the CAP site, and the other at position -557, 192 bp upstream of the TATA box and 213 bp upstream of the CAP site.

The positions of the CAP site, TATA- and CCAAT-box correspond well to the positions found in other eukaryotic polymerase II promoters (32-33)

<u>α-amylase promoter deletions</u>

Plasmid from a large scale preparation of the EH8 subclone was banded twice on a CsCl gradient (see Materials and Methods) to obtain pure supercoiled DNA. Running a sample of the plasmid DNA on an agarose gel showed that at least 85% of the preparation was supercoiled. Then the EH8 plasmid was digested with Bst XI which creates a 3' overhang and with BamHI which creates a 5' overhang end, and care was taken to be sure that the digests were complete. An ExoIII/Mung Bean treatment was done as described in Materials and Methods, and aliquots were taken at 0, 1, 2, 3, 4, 5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5, 10 and 10.5 min. The different mixtures now containing the nested deletions of the EH8 plasmid were used directly in ligation and transformation as explained in Materials and Methods. A whole range of deletion subclones were obtained, these are shown in figure 4. They were all sequenced with the T3 and T7 primers (see figure 8) to locate their 5' end in proportion to the α-Amy 3 promoter. The individual start positions are shown in figure 2A where the arrows indicate the start and extent of the sequence reactions of the deleted subclones.

Three of the nested deletion subclones were chosen for functional analysis, these were 4-1, 6-15 and 8.5-E (indicated with an asterisk in figure 2B).

A large scale plasmid preparation was generated from each of the selected deletions cloned (4-1, 6-15 and 8.5-E) and from the EH8 clone. These were then digested with SacI which cuts in the polylinker of pBSK- and the site blunted (see Materials and Methods) before they were digested with *Sal* I, which cuts on the opposite site of the promoter insert.

A pBI101 plasmid (see Materials and Methods) containing a promotorless ß-glucuronidase (GUS) gene was completely digested with *Hind*III and the *Hind*III sites blunted as described in Materials and Methods. Afterwards the open plasmid was digested with *Sal* I thereby creating a pBI101 *Hind*III ^{Blunt}/*Sal* I vector.

Subcloning and transformation

The Sac I Blunt/*Sal* I fragments obtained from the 4-1, 6-15, 8.5-E and EH8 clones were then subcloned into the pBI101 *Hind*III^{Blunt}/*Sal*I vector and the ligated plasmids transformed into the *Agrobacterium* strain LBA4404 (see Materials and Methods). The colonies obtained were tested using the restriction enzymes *Pst*I and *Sal*I which cut on either side of the inserted fragment. Clones which contained an insert of the right size were then analysed by sequencing with a primer named #589 (see figure 6). The #589 primer primes within the GUS gene of pBI101 and allows the reading of sequences upstream of the promoterless GUS gene thereby covering the inserted promoter deletions.

The pBI101 plasmids containing the selected promoter deletions were named EH, HFP4, HFP6 and HFP8.

In addition, a Southern transfer of the *Pst*I and *Sal* I digested plasmids was hybridized with a labelled insert from the EH8 clone which contained the largest fragment of the α-Amy 3 promoter region, to verify the origin of the inserts.

To produce a smaller fragment than the one covered by 4-1, 6-15, 8.5-E or EH8, another subclone was created, the HE subclone. This was accomplished by digesting the EH8 subclone with *Eco*RI and blunt end the *Eco*RI site followed by a digest of *Hind*III and then isolate the 288 bp fragment containing 3'end promoter sequences (see figure 2B for position of the HE fragment). For subcloning this *Hind*III/*Eco*RI^{Blunt} fragment into a pBI101 vector digested with SmaI and *Hind*III was used in the ligation reaction. The resulting plasmid is called HE and was transformed into the *Agrobacterium* strain LBA4404 (see Materials and Methods). The colonies obtained

on kanamycin plates were tested by digesting purified plasmid with the restriction enzymes *Hind*III and SnaBI. Plasmids from selected colonies were subjected to sequence analysis with the #589 primer as explained above.

In total, five deletions of the α-Amy 3 promoter constructs have been made as explained in the preceding sctions. They cover 1350 bp (EH8), 853 bp (HFP4), 672 bp (HFP6), 506 bp (HFP8) and 288 bp (HE) of the sequences upstream of the *EcoR*I site 5' to the ATG codon (see figure 2 and figure 5). They were cloned in front of the promoterless GUS gene of the pBI101 vector (see Materials and Methods).

Transformation of potato with the promoter constructs

Six LBA4404 colonies containing the 5 deletion constructs and the pBI101, were selected and used for transformation of *Saturna* stem tissue as described in Materials and Methods.

As a negative control some *Saturna* explants were incubated with nontransformed LBA4404 bacteria and nontransformed shoots obtained from selection plates without kanamycin. As a positive control some *Saturna* explants were incubated with LBA4404 previously transformed with the pBI121 plasmid. pBI121 contain a GUS cassette controlled by the Cauliflower mosaic virus (CaMV) 35S promoter which is constitutively expressed in most plant tissues (34–38).

All regenerated shoots after the first (22 days) and second (49 days) harvest were discarded. After 68 days were 40 shoots from each deletion construct, 10 shoot from the negative control and 15 shoots from the positive control harvested and transferred to root induction medium (see Materials and Methods). Each shoots represents, putatively, an individual transformation event and will in the following be referred to as lines. Each line, if the plant is transformed, represent an independent transformation event.

Expression of GUS in leaves of putatively transformed lines

Leaves from the regenerated lines were all GUS tested after root formation. An expression analysis of the α-amylase genes of the present invention revealed that the α-amylase type 3/4 is expressed in tuber-, sprout-, stem- and root tissue but no expression was found in leaves.

The GUS testing of leaves, from the putatively transformed deletion lines, from the lines transformed with the pBI101 plasmid, and from the nontransformed control lines, revealed that there were no GUS activity in any of these. In contrast, GUS testing of plants transformed with the positive control plasmid pBI121 showed GUS expression in the leaves in nearly all the plants (see table 1).

Expression of GUS in microtubers and sprouts

Micro tubers from the lines described above were generated as described in Materials and Methods. These microtubers were examined for their GUS activity and lines containing the EH8, HFP4 and HFP6 deletion constructs showed positive GUS staining. Again the pBI121 control lines gave GUS positive microtubers while the lines containing the pBI101 plasmid showed negative microtubers. Also non-transformed lines microtubers showed no GUS activity as well as lines transformed with the deletion constructs HFP8 and HE.

Sprouts generated from microtubers (see Materials and Methods) were also GUS analysed and only the lines transformed with the pBI121 (positive control) showed GUS activity.

The results are summarized in Table 1, shown below.

TABLE 1

Expression of GUS

Plants TD with:	Leaf of explant	Micro-tuber	Leaf-like of EP	Stem-like of EP	Sprout pot grown plant	Leaf of tubers	Pot grown
EH	0 (36)*	7 (36)	0 (15)	0 (15)	0 (33)	0 (40)	16 (22)
HFP4	0 (36)	2 (36)	0 (15)	0 (15)	0 (31)	0 (40)	15 (24)
HFP6	0 (32)	5 (32)	0 (15)	0 (15)	0 (26)	0 (40)	10 (26)
HFP8	0 (30)	0 (30)	0 (10)	0 (10)	0 (15)	0 (40)	0 (21)
HE	0 (34)	0 (34)	0 (14)	0 (14)	0 (27)	0 (40)	0 (23)
pBI 101	0 (36)	0 (36)	0 (36)	0 (36)	0 (36)	0 (40)	0 (24)
pBI 121	10(15)	10 (15)	10 (10)	10 (10)	6 (6)	10 (15)	5 (5)
Non TD plants	0 (15)	0 (9)	- - -	- - -	0 (9)	0 (15)	0 (12)

* The numbers in brackets are the total numbers of lines tested.

(TD= transformed; EP = Explant)

Expression of GUS in other parts of the planlets

When microtubers are generated they are formed at the end of the stem explant. On top of the explant most often two leaf-like structures are formed. These stem-like explants and the leaf-like tops were examined for GUS activity. As summarized in table 1, none of the deletions containing lines showed any GUS activity in either the stem-or leaf-like tissues of the explants.

All the pBI121 lines which showed GUS activity in their microtuber also had GUS activity in the stem- and leaf-like structures of the explant.

Expression of GUS in leaves, roots, stems and tubers of pot grown lines

The regenerated potato lines were also grown in pots in a growth chamber (at 22°C, 16h light and 15°C, 8h dark) and leaves, roots, stems and tubers were GUS analysed. None of the plant lines, except the control lines containing the pBI121 construction, showed GUS activity in the leaves as summarized in Table 1 and Table 2.

Investigation of GUS expression in tubers harvested from the pot grown plants, repeated the pattern already seen with the lines tested in the microtubers.

The plants carrying one of three constructs EH8, HFP4 and HFP6 showed GUS activity in the tubers while the plants carring the HFP8, HE or pBI101 constructs showed no GUS activity in their tubers.

Again the plants carring the pBI121 construct had GUS activity in their pot grown tubers as expected.

A GUS analysis of the lines listed in table 2 (except for the positive control plants carring pBI121) showed that there were no GUS activity found in root, stem or leaf tissues even though the lines EH8, HFP4 and HFP6 containing lines clearly showed GUS activity in both microtuber and pot grown tubers.

TABLE 2

TABLE 2 Expression of GUS in pot grown potatoes

Line No.	Construct	Stem	Leaf	Root	Tuber
Saturna					
control	- - - -	0	0	0	0
	EH8				
K702-15.2		0	0	0	+
K702-41.6		0	0	0	+
K702-47.3		0	0	0	+
K702-28.2		0	0	0	+
	HFP4				
K699-2.2		0	0	0	+
K699-31.5		0	0	0	+
K699-44.2		0	0	0	+
	HFP6				
K700-1.5		0	0	0	+
K700-24.3		0	0	0	+
K700-38.2		0	0	0	+
	HFP8				
K703-44.6		0	0	0	0
	HE				
K701-5.3		0	0	0	0
K701-15.2		0	0	0	0
K701-18.2		0	0	0	0
K701-16.2		0	0	0	0
K701-49.2		0	0	0	0
	pBI121				
K661-10.4		+	+	+	+
K661-15.3		+	+	+	+

In conclusion, it is clear that none of the α-Amy 3 promoter deletion constructs covering 1534 bp upstream of the ATG initiation codon leads to expression of an otherwise promoterless GUS gene in leaves of plantlets or pot grown plants, in leaf-and stem-like tissues of microtuber explants or in roots and stems of pot grown plants.

GUS expression is only found in microtubers and pot grown tubers clearly showing that this α-Amy 3 promoter contains a tuber specific element clearly separable from the stem, sprout and root expressing element(s) situated upstream of the EH8 deletions 5' end.

In addition this invention also shows that the tuber specific element is situated near and upstream of the HFP8 deletions 5'end and is covered by the delta sequence. This invention also show that the stem, sprout and root expressing element(s) is positionen upstream of the 5' end of EH8, since neither EH8, HFP4, HFP6, HFP8 nor EH constructs gave GUS expression in these tissues.

It is therefore concluded that the elements directing root-specific, stem-specific and sprout-specific expression are located far upstream in the 351 promoter.

The applicability of the promoters is widespread. With the promoters it is possible to direct the expression of proteins into different tissues in the potato plant. It is even possible to direct the expression of proteins into different tissues in other dicot plants.

pJK4

The potato α-amylase encoding sequences originate from plasmid pAmyZ4 (see detailed description in EP-B-0470145). Briefly pAmyZ4 encodes a 407 amino acid long potato α-amylase precursor and in addition contains 149 bp 5' and 201 bp 3' untranslated sequences positioned in the *EcoR*I site of the plasmid pBSK-'s polylinker.

The antisense α-amylase construction pJK4, containing the sequence shown as SEQ. I.D. No. 19, was made by using the SacI and *EcoR*V fragment from pAMYZ4 and subcloning it into an appropriate plasmid - such as SmaI and SacI digested pEPL plasmid (see Figure

10). This places the antisense sequence downstream of an enhanced 35S promoter (E35S) and upstream of the DW2t terminator. This plasmid is called pEPLZ4Sac-Eco and a partial *Hind*III fragment containing the E35S promoter, the antisense potato sequence and the DW2t terminator was further subcloned into a *Hind*III digested pBI121, thereby creating the binary plasmid pJK4, see figure 9.

Other modifications of the present invention will be apparent to those skilled in the art without departing from the scope of the invention.

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SEQUENCE LISTING

(1) GENERAL INFORMATION

NAME OF APPLICANTS: DANISCO A/S
BUSINESS ADDRESS: Langebrogade 1
DK-1001 Copenhagen K
Denmark

TITLE OF INVENTION: PROMOTER

(2a) INFORMATION FOR SEQUENCE I.D. 1
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 166 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
        10         20         30         40
ATAGCTTGAG GCGAAAATAT TTAATAAAAA CACTTCTTAA

        50         60         70         80
TTTGTTTATA TGTTCAATTG AACATGTCCG TGATTAGAAA

        90        100        110        120
ATTAAATTAA ATTCAATGAC AAATTTAATA ATTTGACACA

       130        140        150        160
AAATTTATGA AAAAAATATC AAAATATAAA GAAATATTTT

TTTTGA
```

(2b) INFORMATION FOR SEQUENCE I.D. 2
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 291 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
        10         20         30         40
AAGCTTCCAA TGAACCGTTG CCATGTGTCA CTGCCTATTC

        50         60         70         80
ACCGCGAAAC ATGAATATCA CTGACGAACG ATTTCGGAGC

        90        100        110        120
GGAACGAATC CAGAAAATGG ATTACTTTCT ATAAATTCCT
```

```
       130         140         150         160
CGAATCTCAA CTCCATTTCG TAAAAATAAA ATTAAAAATA

       170         180         190         200
TTGTTTCTTT TTGTATTTCT TTTTGTATTT CTGGTTTATG

       210         220         230         240
TGGTGATCGA ATTTTCAATT TTTTTACTGG TAGTGATTCC

       250         260         270         280
TACTTTTCTT CAATTGCATT TCTCCTTTTT CCATTTCACG

       290
GTTGAGAATT C
```

(2c) INFORMATION FOR SEQUENCE I.D. 3
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 508 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
AATGGATTAA AAAGAAAAAA AAAACAAATA AATTGAACCG  40
GGATAAGTTG GTTGTTTAAT TGATTATTGA TTATGATCTC  80
AATTTGACAT TTTGCGCGAT CTTTCGACCT CAATTCGTAT 120
GAACTGACAC TACGCCAATG GACAGTCGCC GTCGTCACCG 160
CCACCGCACT ATTCTCGACG CGTCGTCTAT CTCCTCCACC 200
CCACAGCCGT CAATTCCAAG CTTCCAATGA ACCGTTGCCA 240
TGTGTCACTG CCTATTCACC GCGAAACATG AATATCACTG 280
ACGAACGATT TCGGAGCGGA ACGAATCCAG AAAATGGATT 320
ACTTTCTATA AATTCCTCGA ATCTCAACTC CATTTCGTAA 360
AAATAAAATT AAAAATATTG TTTCTTTTTG TATTTCTTTT 400
TGTATTTCTG GTTTATGTGG TGATCGAATT TTCAATTTTT 440
TTACTGGTAG TGATTCCTAC TTTTCTTCAA TTGCATTTCT 480
CCTTTTTCCA TTTCACGGTT GAGAATTC              508
```

(2d) INFORMATION FOR SEQUENCE I.D. 4
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 514 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
                          TTTT 04
GAAATGGATT AAAAAGAAAA AAAAAACAAA TAAATTGAAC  44
CGGGATAAGT TGGTTGTTTA ATTGATTATT GATTATGATC  84
TCAATTTGAC ATTTTGCGCG ATCTTTCGAC CTCAATTCGT 124
ATGAACTGAC ACTACGCCAA TGGACAGTCG CCGTCGTCAC 164
CGCCACCGCA CTATTCTCGA CGCGTCGTCT ATCTCCTCCA 204
```

CCCCACAGCC GTCAATTCCA AGCTTCCAAT GAACCGTTGC 244
CATGTGTCAC TGCCTATTCA CCGCGAAACA TGAATATCAC 284
TGACGAACGA TTTCGGAGCG GAACGAATCC AGAAAATGGA 324
TTACTTTCTA TAAATTCCTC GAATCTCAAC TCCATTTCGT 364
AAAAATAAAA TTAAAAATAT TGTTTCTTTT TGTATTTCTT 404
TTTGTATTTC TGGTTTATGT GGTGATCGAA TTTTCAATTT 444
TTTTACTGGT AGTGATTCCT ACTTTTCTTC AATTGCATTT 484
CTCCTTTTTC CATTTCACGG TTGAGAATTC 510

(2e) INFORMATION FOR SEQUENCE I.D. 5
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 518 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

TTTTTTTTGA AATGGATTAA AAAGAAAAAA AAAACAAATA 40
AATTGAACCG GGATAAGTTG GTTGTTTAAT TGATTATTGA 80
TTATGATCTC AATTTGACAT TTTGCGCGAT CTTTCGACCT 120
CAATTCGTAT GAACTGACAC TACGCCAATG GACAGTCGCC 160
GTCGTCACCG CCACCGCACT ATTCTCGACG CGTCGTCTAT 200
CTCCTCCACC CCACAGCCGT CAATTCCAAG CTTCCAATGA 240
ACCGTTGCCA TGTGTCACTG CCTATTCACC GCGAAACATG 280
AATATCACTG ACGAACGATT TCGGAGCGGA ACGAATCCAG 320
AAAATGGATT ACTTTCTATA AATTCCTCGA ATCTCAACTC 360
CATTTCGTAA AAATAAAATT AAAAATATTG TTTCTTTTTG 400
TATTTCTTTT TGTATTTCTG GTTTATGTGG TGATCGAATT 440
TTCAATTTTT TTACTGGTAG TGATTCCTAC TTTTCTTCAA 480
TTGCATTTCT CCTTTTTCCA TTTCACGGTT GAGAATTC 518

(2f) INFORMATION FOR SEQUENCE I.D. 6
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 631 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

GTTTATATGT TCAATTGAAC ATGTCCGTGA TTAGAAAATT 40
AAATTAAATT CAATGACAAA TTTAATAATT TGACACAAAA 80
TTTATGAAAA AAATATCAAA ATATAAAGAA ATATTTTTTT 120
TGAAATGGAT TAAAAAGAAA AAAAAAACAA ATAAATTGAA 160
CCGGGATAAG TTGGTTGTTT AATTGATTAT TGATTATGAT 200
CTCAATTTGA CATTTTGCGC GATCTTTCGA CCTCAATTCG 240
TATGAACTGA CACTACGCCA ATGGACAGTC GCCGTCGTCA 280
CCGCCACCGC ACTATTCTCG ACGCGTCGTC TATCTCCTCC 320
ACCCCACAGC CGTCAATTCC AAGCTTCCAA TGAACCGTTG 360
CCATGTGTCA CTGCCTATTC ACCGCGAAAC ATGAATATCA 400
CTGACGAACG ATTTCGGAGC GGAACGAATC CAGAAAATGG 440
ATTACTTTCT ATAAATTCCT CGAATCTCAA CTCCATTTCG 480

TAAAAATAAA ATTAAAAATA TTGTTTCTTT TTGTATTTCT 520
TTTTGTATTT CTGGTTTATG TGGTGATCGA ATTTTCAATT 560
TTTTTACTGG TAGTGATTCC TACTTTTCTT CAATTGCATT 600
TCTCCTTTTT CCATTTCACG GTTGAGAATT C 631

(2g) INFORMATION FOR SEQUENCE I.D. 7
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 674 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

ATAGCTTGAG GCGAAAATAT TTAATAAAAA CACTTCTTAA 40
TTTGTTTATA TGTTCAATTG AACATGTCCG TGATTAGAAA 80
ATTAAATTAA ATTCAATGAC AAATTTAATA ATTTGACACA 120
AAATTTATGA AAAAAATATC AAAATATAAA GAAATATTTT 160
TTTTGAAATG GATTAAAAAG AAAAAAAAAA CAAATAAATT 200
GAACCGGGAT AAGTTGGTTG TTTAATTGAT TATTGATTAT 240
GATCTCAATT TGACATTTTG CGCGATCTTT CGACCTCAAT 280
TCGTATGAAC TGACACTACG CCAATGGACA GTCGCCGTCG 320
TCACCGCCAC CGCACTATTC TCGACGCGTC GTCTATCTCC 360
TCCACCCCAC AGCCGTCAAT TCCAAGCTTC CAATGAACCG 400
TTGCCATGTG TCACTGCCTA TTCACCGCGA AACATGAATA 440
TCACTGACGA ACGATTTCGG AGCGGAACGA ATCCAGAAAA 480
TGGATTACTT TCTATAAATT CCTCGAATCT CAACTCCATT 520
TCGTAAAAAT AAAATTAAAA ATATTGTTTC TTTTTGTATT 560
TCTTTTTGTA TTTCTGGTTT ATGTGGTGAT CGAATTTTCA 600
ATTTTTTTAC TGGTAGTGAT TCCTACTTTT CTTCAATTGC 640
ATTTCTCCTT TTTCCATTTC ACGGTTGAGA ATTC 674

(2h) INFORMATION FOR SEQUENCE I.D. 8
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 687 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

TTCCTTTCCT CATATAGCTT GAGGCGAAAA TATTTAATAA 40
AAACACTTCT TAATTTGTTT ATATGTTCAA TTGAACATGT 80
CCGTGATTAG AAAATTAAAT TAAATTCAAT GACAAATTTA 120
ATAATTTGAC ACAAAATTTA TGAAAAAAAT ATCAAAATAT 160
AAAGAAATAT TTTTTTTGAA ATGGATTAAA AAGAAAAAAA 200
AAACAAATAA ATTGAACCGG GATAAGTTGG TTGTTTAATT 240
GATTATTGAT TATGATCTCA ATTTGACATT TTGCGCGATC 280
TTTCGACCTC AATTCGTATG AACTGACACT ACGCCAATGG 320
ACAGTCGCCG TCGTCACCGC CACCGCACTA TTCTCGACGC 360
GTCGTCTATC TCCTCCACCC CACAGCCGTC AATTCCAAGC 400
TTCCAATGAA CCGTTGCCAT GTGTCACTGC CTATTCACCG 440
CGAAACATGA ATATCACTGA CGAACGATTT CGGAGCGGAA 480
CGAATCCAGA AAATGGATTA CTTTCTATAA ATTCCTCGAA 520

TCTCAACTCC ATTTCGTAAA AATAAAATTA AAAATATTGT 560
TTCTTTTTGT ATTTCTTTTT GTATTTCTGG TTTATGTGGT 600
GATCGAATTT TCAATTTTTT TACTGGTAGT GATTCCTACT 640
TTTCTTCAAT TGCATTTCTC CTTTTTCCAT TTCACGGTTG 680
AGAATTC 687

(2i) INFORMATION FOR SEQUENCE I.D. 9
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 693 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

CACTGATTCC TTTCCTCATA TAGCTTGAGG CGAAAATATT 40
TAATAAAAAC ACTTCTTAAT TTGTTTATAT GTTCAATTGA 80
ACATGTCCGT GATTAGAAAA TTAAATTAAA TTCAATGACA 120
AATTTAATAA TTTGACACAA AATTTATGAA AAAAATATCA 160
AAATATAAAG AAATATTTTT TTTGAAATGG ATTAAAAAGA 200
AAAAAAAAAC AAATAAATTG AACCGGGATA AGTTGGTTGT 240
TTAATTGATT ATTGATTATG ATCTCAATTT GACATTTTGC 280
GCGATCTTTC GACCTCAATT CGTATGAACT GACACTACGC 320
CAATGGACAG TCGCCGTCGT CACCGCCACC GCACTATTCT 360
CGACGCGTCG TCTATCTCCT CCACCCCACA GCCGTCAATT 400
CCAAGCTTCC AATGAACCGT TGCCATGTGT CACTGCCTAT 440
TCACCGCGAA ACATGAATAT CACTGACGAA CGATTTCGGA 480
GCGGAACGAA TCCAGAAAAT GGATTACTTT CTATAAATTC 520
CTCGAATCTC AACTCCATTT CGTAAAAATA AAATTAAAAA 560
TATTGTTTCT TTTTGTATTT CTTTTTGTAT TTCTGGTTTA 600
TGTGGTGATC GAATTTTCAA TTTTTTTACT GGTAGTGATT 640
CCTACTTTTC TTCAATTGCA TTTCTCCTTT TTCCATTTCA 680
CGGTTGAGAA TTC 693

(2j) INFORMATION FOR SEQUENCE I.D. 10
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 758 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

CTTGCGCCTT TCCCTAAATT AAGTAAAACT CTTCGCCTCA 40
TGCCTTACGC CTCCGCCTTT TAAAACACTG ATTCCTTTCC 80
TCATATAGCT TGAGGCGAAA ATATTTAATA AAAACACTTC 120
TTAATTTGTT TATATGTTCA ATTGAACATG TCCGTGATTA 160
GAAAATTAAA TTAAATTCAA TGACAAATTT AATAATTTGA 200
CACAAAATTT ATGAAAAAAA TATCAAAATA TAAAGAAATA 240
TTTTTTTTGA AATGGATTAA AAAGAAAAAA AAAACAAATA 280
AATTGAACCG GGATAAGTTG GTTGTTTAAT TGATTATTGA 320
TTATGATCTC AATTTGACAT TTTGCGCGAT CTTTCGACCT 360

CAATTCGTAT GAACTGACAC TACGCCAATG GACAGTCGCC 400
GTCGTCACCG CCACCGCACT ATTCTCGACG CGTCGTCTAT 440
CTCCTCCACC CCACAGCCGT CAATTCCAAG CTTCCAATGA 480
ACCGTTGCCA TGTGTCACTG CCTATTCACC GCGAAACATG 520
AATATCACTG ACGAACGATT TCGGAGCGGA ACGAATCCAG 560
AAAATGGATT ACTTTCTATA AATTCCTCGA ATCTCAACTC 600
CATTTCGTAA AAATAAAATT AAAAATATTG TTTCTTTTTG 640
TATTTCTTTT TGTATTTCTG GTTTATGTGG TGATCGAATT 680
TTCAATTTTT TTACTGGTAG TGATTCCTAC TTTTCTTCAA 720
TTGCATTTCT CCTTTTTCCA TTTCACGGTT GAGAATTC 758

(2k) INFORMATION FOR SEQUENCE I.D. 11
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 855 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

CAAATTTTGA TGTATTTTTA TAATTTTGTA TTATTATATT 40
ATTATACTAT ATTTAAAAAT TTAAAGATCC ATAGGGCTTA 80
CGCCCCACGT CAAGAGGCTT GCGCCTTTCC CTAAATTAAG 120
TAAAACTCTT CGCCTCATGC CTTACGCCTC CGCCTTTTAA 160
AACACTGATT CCTTTCCTCA TATAGCTTGA GGCGAAAATA 200
TTTAATAAAA ACACTTCTTA ATTTGTTTAT ATGTTCAATT 240
GAACATGTCC GTGATTAGAA AATTAAATTA AATTCAATGA 280
CAAATTTAAT AATTTGACAC AAAATTTATG AAAAAAATAT 320
CAAAATATAA AGAAATATTT TTTTTGAAAT GGATTAAAAA 360
GAAAAAAAAA ACAAATAAAT TGAACCGGGA TAAGTTGGTT 400
GTTTAATTGA TTATTGATTA TGATCTCAAT TTGACATTTT 440
GCGCGATCTT TCGACCTCAA TTCGTATGAA CTGACACTAC 480
GCCAATGGAC AGTCGCCGTC GTCACCGCCA CCGCACTATT 520
CTCGACGCGT CGTCTATCTC CTCCACCCCA CAGCCGTCAA 560
TTCCAAGCTT CCAATGAACC GTTGCCATGT GTCACTGCCT 600
ATTCACCGCG AAACATGAAT ATCACTGACG AACGATTTCG 640
GAGCGGAACG AATCCAGAAA ATGGATTACT TTCTATAAAT 680
TCCTCGAATC TCAACTCCAT TTCGTAAAAA TAAAATTAAA 720
AATATTGTTT CTTTTTGTAT TTCTTTTTGT ATTTCTGGTT 760
TATGTGGTGA TCGAATTTTC AATTTTTTTA CTGGTAGTGA 800
TTCCTACTTT TCTTCAATTG CATTTCTCCT TTTTCCATTT 840
CACGGTTGAG AATTC 855

(21) INFORMATION FOR SEQUENCE I.D. 12
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 859 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

ATTTCAAATT TTGATGTATT TTTATAATTT TGTATTATTA 40
TATTATTATA CTATATTTAA AAATTTAAAG ATCCATAGGG 80

```
CTTACGCCCC ACGTCAAGAG GCTTGCGCCT TTCCCTAAAT 120
TAAGTAAAAC TCTTCGCCTC ATGCCTTACG CCTCCGCCTT 160
TTAAAACACT GATTCCTTTC CTCATATAGC TTGAGGCGAA 200
AATATTTAAT AAAAACACTT CTTAATTTGT TTATATGTTC 240
AATTGAACAT GTCCGTGATT AGAAAATTAA ATTAAATTCA 280
ATGACAAATT TAATAATTTG ACACAAAATT TATGAAAAAA 320
ATATCAAAAT ATAAAGAAAT ATTTTTTTTG AAATGGATTA 360
AAAAGAAAAA AAAAACAAAT AAATTGAACC GGGATAAGTT 400
GGTTGTTTAA TTGATTATTG ATTATGATCT CAATTTGACA 440
TTTTGCGCGA TCTTTCGACC TCAATTCGTA TGAACTGACA 480
CTACGCCAAT GGACAGTCGC CGTCGTCACC GCCACCGCAC 520
TATTCTCGAC GCGTCGTCTA TCTCCTCCAC CCCACAGCCG 560
TCAATTCCAA GCTTCCAATG AACCGTTGCC ATGTGTCACT 600
GCCTATTCAC CGCGAAACAT GAATATCACT GACGAACGAT 640
TTCGGAGCGG AACGAATCCA GAAAATGGAT TACTTTCTAT 680
AAATTCCTCG AATCTCAACT CCATTTCGTA AAAATAAAAT 720
TAAAAATATT GTTTCTTTTT GTATTTCTTT TTGTATTTCT 760
GGTTTATGTG GTGATCGAAT TTTCAATTTT TTTACTGGTA 800
GTGATTCCTA CTTTTCTTCA ATTGCATTTC TCCTTTTTCC 840
ATTTCACGGT TGAGAATTC                       859
```

u(2m) INFORMATION FOR SEQUENCE I.D. 13
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1214 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
GAAGGTGATT ATACATTACG TAACATTTCT TTTAAAAATA  40
TGTAAGCAAA TTTACTTTTT AACTTATCAT TGATCTTCAT  80
GGTTTTGTCA TAAATCTCAA AGTTATCATA TTTTATATAG 120
CTATTTGAAA GTAATTTTAT TTTTACTCAT CATTGAGTGA 160
TGCTTTTATT ATAATACTAG TAAGTTTTAT TTATTATTTT 200
CTTTTAGGGG TGAATTGTAT AATATAATAA AAAATATATT 240
TTTAGAAATA ATGATTCTTT TATTATTAAA AAGTTAAGAT 280
ATTAGATTAT TTATGCTTGT ATAATAATGA ACGAAGTTTT 320
ATTTTCTATG AGTTTCATTA ATCATGTTTG TAATTATTTC 360
AAATTTTGAT GTATTTTTAT AATTTTGTAT TATTATATTA 400
TTATACTATA TTTAAAAATT TAAAGATCCA TAGGGCTTAC 440
GCCCCACGTC AAGAGGCTTG CGCCTTTCCC TAAATTAAGT 480
AAAACTCTTC GCCTCATGCC TTACGCCTCC GCCTTTTAAA 520
ACACTGATTC CTTTCCTCAT ATAGCTTGAG GCGAAAATAT 560
TTAATAAAAA CACTTCTTAA TTTGTTTATA TGTTCAATTG 600
AACATGTCCG TGATTAGAAA ATTAAATTAA ATTCAATGAC 640
AAATTTAATA ATTTGACACA AAATTTATGA AAAAAATATC 680
AAAATATAAA GAAATATTTT TTTTGAAATG GATTAAAAAG 720
AAAAAAAAAA CAAATAAATT GAACCGGGAT AAGTTGGTTG 760
TTTAATTGAT TATTGATTAT GATCTCAATT TGACATTTTG 800
CGCGATCTTT CGACCTCAAT TCGTATGAAC TGACACTACG 840
CCAATGGACA GTCGCCGTCG TCACCGCCAC CGCACTATTC 880
TCGACGCGTC GTCTATCTCC TCCACCCCAC AGCCGTCAAT 920
TCCAAGCTTC CAATGAACCG TTGCCATGTG TCACTGCCTA 960
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TTCACCGCGA AACATGAATA TCACTGACGA ACGATTTCGG 1000
AGCGGAACGA ATCCAGAAAA TGGATTACTT TCTATAAATT 1040
CCTCGAATCT CAACTCCATT TCGTAAAAAT AAAATTAAAA 1080
ATATTGTTTC TTTTTGTATT TCTTTTTGTA TTTCTGGTTT 1120
ATGTGGTGAT CGAATTTTCA ATTTTTTTAC TGGTAGTGAT 1160
TCCTACTTTT CTTCAATTGC ATTTCTCCTT TTTCCATTTC 1200
ACGGTTGAGA ATTC 1214

(2n) INFORMATION FOR SEQUENCE I.D. 14
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1232 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

ACTATTTGAT AACATTATGA AGGTGATTAT ACATTACGTA 40
ACATTTCTTT TAAAAATATG TAAGCAAATT TACTTTTTAA 80
CTTATCATTG ATCTTCATGG TTTTGTCATA AATCTCAAAG 120
TTATCATATT TTATATAGCT ATTTGAAAGT AATTTTATTT 160
TTACTCATCA TTGAGTGATG CTTTTATTAT AATACTAGTA 200
AGTTTTATTT ATTATTTTCT TTTAGGGGTG AATTGTATAA 240
TATAATAAAA AATATATTTT TAGAAATAAT GATTCTTTTA 280
TTATTAAAAA GTTAAGATAT TAGATTATTT ATGCTTGTAT 320
AATAATGAAC GAAGTTTTAT TTTCTATGAG TTTCATTAAT 360
CATGTTTGTA ATTATTTCAA ATTTTGATGT ATTTTTATAA 400
TTTTGTATTA TTATATTATT ATACTATATT TAAAAATTTA 440
AAGATCCATA GGGCTTACGC CCCACGTCAA GAGGCTTGCG 480
CCTTTCCCTA AATTAAGTAA AACTCTTCGC CTCATGCCTT 520
ACGCCTCCGC CTTTTAAAAC ACTGATTCCT TTCCTCATAT 560
AGCTTGAGGC GAAAATATTT AATAAAAACA CTTCTTAATT 600
TGTTTATATG TTCAATTGAA CATGTCCGTG ATTAGAAAAT 640
TAAATTAAAT TCAATGACAA ATTTAATAAT TTGACACAAA 680
ATTTATGAAA AAAATATCAA AATATAAAGA AATATTTTTT 720
TTGAAATGGA TTAAAAAGAA AAAAAAAACA AATAAATTGA 760
ACCGGGATAA GTTGGTTGTT TAATTGATTA TTGATTATGA 800
TCTCAATTTG ACATTTTGCG CGATCTTTCG ACCTCAATTC 840
GTATGAACTG ACACTACGCC AATGGACAGT CGCCGTCGTC 880
ACCGCCACCG CACTATTCTC GACGCGTCGT CTATCTCCTC 920
CACCCCACAG CCGTCAATTC CAAGCTTCCA ATGAACCGTT 960
GCCATGTGTC ACTGCCTATT CACCGCGAAA CATGAATATC 1000
ACTGACGAAC GATTTCGGAG CGGAACGAAT CCAGAAAATG 1040
GATTACTTTC TATAAATTCC TCGAATCTCA ACTCCATTTC 1080
GTAAAAATAA AATTAAAAAT ATTGTTTCTT TTTGTATTTC 1120
TTTTTGTATT TCTGGTTTAT GTGGTGATCG AATTTTCAAT 1160
TTTTTTACTG GTAGTGATTC CTACTTTTCT TCAATTGCAT 1200
TTCTCCTTTT TCCATTTCAC GGTTGAGAAT TC 1232

(2o) INFORMATION FOR SEQUENCE I.D. 15
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1352 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
 GGCCTCACA TCAACCTTCA TAATTCTTGA ATGAATGAAT  39
GATAGACTTC ATAATTTTTT AACCTATACA TATAAGAAAA  79
TTGAGAGTAA CTCAAATAAC AAGTTGTAGT ATCACATCTT 119
TACTATTTGA TAACATTATG AAGGTGATTA TACATTACGT 159
AACATTTCTT TTAAAAATAT GTAAGCAAAT TTACTTTTTA 199
ACTTATCATT GATCTTCATG GTTTTGTCAT AAATCTCAAA 239
GTTATCATAT TTTATATAGC TATTTGAAAG TAATTTTATT 279
TTTACTCATC ATTGAGTGAT GCTTTTATTA TAATACTAGT 319
AAGTTTTATT TATTATTTTC TTTTAGGGGT GAATTGTATA 359
ATATAATAAA AAATATATTT TTAGAAATAA TGATTCTTTT 399
ATTATTAAAA AGTTAAGATA TTAGATTATT TATGCTTGTA 439
TAATAATGAA CGAAGTTTTA TTTTCTATGA GTTTCATTAA 479
TCATGTTTGT AATTATTTCA AATTTTGATG TATTTTTATA 519
ATTTTGTATT ATTATATTAT TATACTATAT TTAAAAATTT 559
AAAGATCCAT AGGGCTTACG CCCCACGTCA AGAGGCTTGC 599
GCCTTTCCCT AAATTAAGTA AAACTCTTCG CCTCATGCCT 639
TACGCCTCCG CCTTTTAAAA CACTGATTCC TTTCCTCATA 679
TAGCTTGAGG CGAAAATATT TAATAAAAAC ACTTCTTAAT 719
TTGTTTATAT GTTCAATTGA ACATGTCCGT GATTAGAAAA 759
TTAAATTAAA TTCAATGACA AATTTAATAA TTTGACACAA 799
AATTTATGAA AAAAATATCA AAATATAAAG AAATATTTTT 839
TTTGAAATGG ATTAAAAAGA AAAAAAAAAC AAATAAATTG 879
AACCGGGATA AGTTGGTTGT TTAATTGATT ATTGATTATG 919
ATCTCAATTT GACATTTTGC GCGATCTTTC GACCTCAATT 959
CGTATGAACT GACACTACGC CAATGGACAG TCGCCGTCGT 999
CACCGCCACC GCACTATTCT CGACGCGTCG TCTATCTCCT 1039
CCACCCCACA GCCGTCAATT CCAAGCTTCC AATGAACCGT 1079
TGCCATGTGT CACTGCCTAT TCACCGCGAA ACATGAATAT 1119
CACTGACGAA CGATTTCGGA GCGGAACGAA TCCAGAAAAT 1159
GGATTACTTT CTATAAATTC CTCGAATCTC AACTCCATTT 1199
CGTAAAAATA AAATTAAAAA TATTGTTTCT TTTTGTATTT 1239
CTTTTTGTAT TTCTGGTTTA TGTGGTGATC GAATTTTCAA 1279
TTTTTTTACT GGTAGTGATT CCTACTTTTC TTCAATTGCA 1319
TTTCTCCTTT TTCCATTTCA CGGTTGAGAA TTC        1352
```

(2p) INFORMATION FOR SEQUENCE I.D. 16
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1734 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
         10          20          30          40
TCTTTAAGTT GTTTGCTTGA TTTTTCTTCT TCAATCTTCT

         50          60          70          80
ATATTTAATT CGTTTTAGCT TCAAACTTCT TCAATTTTAT

         90         100         110         120
TTCAATTTAA TTCTACAAAA AAAATCTCTA TTTAGCACCA

        130         140         150         160
TTCATAAAAT TCATGCTCAA AATGGGCAAA CATAAATAAT

        170         180         190         200
AAATGTGAAG TAAATAATGG ATTAAAATAT ATATTTTTGG

        210         220         230         240
GCCTCACATC AACCTTCATA ATTCTTGAAT GAATGAATGA

        250         260         270         280
TAGACTTCAT AATTTTTTAA CCTATACATA TAAGAAAATT

        290         300         310         320
GAGAGTAACT CAAATAACAA GTTGTAGTAT CACATCTTTA

        330         340         350         360
CTATTTGATA ACATTATGAA GGTGATTATA CATTACGTAA

        370         380         390         400
CATTTCTTTT AAAAATATGT AAGCAAATTT ACTTTTTAAC

        410         420         430         440
TTATCATTGA TCTTCATGGT TTTGTCATAA ATCTCAAAGT

        450         460         470         480
TATCATATTT TATATAGCTA TTTGAAAGTA ATTTTATTTT

        490         500         510         520
TACTCATCAT TGAGTGATGC TTTTATTATA ATACTAGTAA

        530         540         550         560
GTTTTATTTA TTATTTTCTT TTAGGGGTGA ATTGTATAAT

        570         580         590         600
ATAATAAAAA ATATATTTTT AGAAATAATG ATTCTTTTAT
```

610 620 630 640
TATTAAAAAG TTAAGATATT AGATTATTTA TGCTTGTATA

650 660 670 680
ATAATGAACG AAGTTTTATT TTCTATGAGT TTCATTAATC

690 700 710 720
ATGTTTGTAA TTATTTCAAA TTTTGATGTA TTTTTATAAT

730 740 750 760
TTTGTATTAT TATATTATTA TACTATATTT AAAAATTTAA

770 780 790 800
AGATCCATAG GGCTTACGCC CCACGTCAAG AGGCTTGCGC

810 820 830 840
CTTTCCCTAA ATTAAGTAAA ACTCTTCGCC TCATGCCTTA

850 860 870 880
CGCCTCCGCC TTTTAAAACA CTGATTCCTT TCCTCATATA

890 900 910 920
GCTTGAGGCG AAAATATTTA ATAAAAACAC TTCTTAATTT

930 940 950 960
GTTTATATGT TCAATTGAAC ATGTCCGTGA TTAGAAAATT

970 980 990 1000
AAATTAAATT CAATGACAAA TTTAATAATT TGACACAAAA

1010 1020 1030 1040
TTTATGAAAA AAATATCAAA ATATAAAGAA ATATTTTTTT

1050 1060 1070 1080
TGAAATGGAT TAAAAAGAAA AAAAAAACAA ATAAATTGAA

1090 1100 1110 1120
CCGGGATAAG TTGGTTGTTT AATTGATTAT TGATTATGAT

1130 1140 1150 1160
CTCAATTTGA CATTTTGCGC GATCTTTCGA CCTCAATTCG

1170 1180 1190 1200
TATGAACTGA CACTACGCCA ATGGACAGTC GCCGTCGTCA

1210 1220 1230 1240
CCGCCACCGC ACTATTCTCG ACGCGTCGTC TATCTCCTCC

1250 1260 1270 1280
ACCCCACAGC CGTCAATTCC AAGCTTCCAA TGAACCGTTG

1290 1300 1310 1320
CCATGTGTCA CTGCCTATTC ACCGCGAAAC ATGAATATCA

```
      1330        1340        1350        1360
CTGACGAACG ATTTCGGAGC GGAACGAATC CAGAAAATGG

      1370        1380        1390        1400
ATTACTTTCT ATAAATTCCT CGAATCTCAA CTCCATTTCG

      1410        1420        1430        1440
TAAAAATAAA ATTAAAAATA TTGTTTCTTT TTGTATTTCT

      1450        1460        1470        1480
TTTTGTATTT CTGGTTTATG TGGTGATCGA ATTTTCAATT

      1490        1500        1510        1520
TTTTTACTGG TAGTGATTCC TACTTTTCTT CAATTGCATT

      1530        1540        1550        1560
TCTCCTTTTT CCATTTCACG GTTGAGAATT CATGATTCCT

      1570        1580        1590        1600
TATCAGAGGA ATCGATCCGA TTTGACTAAT TTCACTTTTC

      1610        1620        1630        1640
GTCTGTATAA ATACCAGAGT ATCTAGGTTG AGGAACGTAA

      1650        1660        1670        1680
TTTCAAGCTG CGATCGGCTT TTTCCCCTGA ACGAGCAAAC

      1690        1700        1710        1720
ACAGGTTGTG GGTTCGAGTT AGCAAGGGAC GTATAATCTC

     1730
AACTACAATC CATT
```

(2q) INFORMATION FOR SEQUENCE I.D. 17
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1920 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
        10          20          30          40
TCTTTAAGTT GTTTGCTTGA TTTTTCTTCT TCAATCTTCT

        50          60          70          80
ATATTTAATT CGTTTTAGCT TCAAACTTCT TCAATTTTAT

        90          100         110         120
TTCAATTTAA TTCTACAAAA AAAATCTCTA TTTAGCACCA

       130         140         150         160
TTCATAAAAT TCATGCTCAA AATGGGCAAA CATAAATAAT
```

```
       170         180         190         200
AAATGTGAAG TAAATAATGG ATTAAAATAT ATATTTTTGG

       210         220         230         240
GCCTCACATC AACCTTCATA ATTCTTGAAT GAATGAATGA

       250         260         270         280
TAGACTTCAT AATTTTTTAA CCTATACATA TAAGAAAATT

       290         300         310         320
GAGAGTAACT CAAATAACAA GTTGTAGTAT CACATCTTTA

       330         340         350         360
CTATTTGATA ACATTATGAA GGTGATTATA CATTACGTAA

       370         380         390         400
CATTTCTTTT AAAAATATGT AAGCAAATTT ACTTTTTAAC

       410         420         430         440
TTATCATTGA TCTTCATGGT TTTGTCATAA ATCTCAAAGT

       450         460         470         480
TATCATATTT TATATAGCTA TTTGAAAGTA ATTTTATTTT

       490         500         510         520
TACTCATCAT TGAGTGATGC TTTTATTATA ATACTAGTAA

       530         540         550         560
GTTTTATTTA TTATTTTCTT TTAGGGGTGA ATTGTATAAT

       570         580         590         600
ATAATAAAAA ATATATTTTT AGAAATAATG ATTCTTTTAT

       610         620         630         640
TATTAAAAAG TTAAGATATT AGATTATTTA TGCTTGTATA

       650         660         670         680
ATAATGAACG AAGTTTTATT TTCTATGAGT TTCATTAATC

       690         700         710         720
ATGTTTGTAA TTATTTCAAA TTTTGATGTA TTTTTATAAT

       730         740         750         760
TTTGTATTAT TATATTATTA TACTATATTT AAAAATTTAA

       770         780         790         800
AGATCCATAG GGCTTACGCC CCACGTCAAG AGGCTTGCGC

       810         820         830         840
CTTTCCCTAA ATTAAGTAAA ACTCTTCGCC TCATGCCTTA

       850         860         870         880
CGCCTCCGCC TTTTAAAACA CTGATTCCTT TCCTCATATA
```

```
       890         900         910         920
GCTTGAGGCG AAAATATTTA ATAAAAACAC TTCTTAATTT

       930         940         950         960
GTTTATATGT TCAATTGAAC ATGTCCGTGA TTAGAAAATT

       970         980         990        1000
AAATTAAATT CAATGACAAA TTTAATAATT TGACACAAAA

      1010        1020        1030        1040
TTTATGAAAA AAATATCAAA ATATAAAGAA ATATTTTTTT

      1050        1060        1070        1080
TGAAATGGAT TAAAAAGAAA AAAAAAACAA ATAAATTGAA

      1090        1100        1110        1120
CCGGGATAAG TTGGTTGTTT AATTGATTAT TGATTATGAT

      1130        1140        1150        1160
CTCAATTTGA CATTTTGCGC GATCTTTCGA CCTCAATTCG

      1170        1180        1190        1200
TATGAACTGA CACTACGCCA ATGGACAGTC GCCGTCGTCA

      1210        1220        1230        1240
CCGCCACCGC ACTATTCTCG ACGCGTCGTC TATCTCCTCC

      1250        1260        1270        1280
ACCCCACAGC CGTCAATTCC AAGCTTCCAA TGAACCGTTG

      1290        1300        1310        1320
CCATGTGTCA CTGCCTATTC ACCGCGAAAC ATGAATATCA

      1330        1340        1350        1360
CTGACGAACG ATTTCGGAGC GGAACGAATC CAGAAAATGG

      1370        1380        1390        1400
ATTACTTTCT ATAAATTCCT CGAATCTCAA CTCCATTTCG

      1410        1420        1430        1440
TAAAAATAAA ATTAAAAATA TTGTTTCTTT TTGTATTTCT

      1450        1460        1470        1480
TTTTGTATTT CTGGTTTATG TGGTGATCGA ATTTTCAATT

      1490        1500        1510        1520
TTTTTACTGG TAGTGATTCC TACTTTTCTT CAATTGCATT

      1530        1540        1550        1560
TCTCCTTTTT CCATTTCACG GTTGAGAATT CATGATTCCT

      1570        1580        1590        1600
TATCAGAGGA ATCGATCCGA TTTGACTAAT TTCACTTTTC
```

```
      1610        1620        1630        1640
GTCTGTATAA ATACCAGAGT ATCTAGGTTG AGGAACGTAA

      1650        1660        1670        1680
TTTCAAGCTG CGATCGGCTT TTTCCCCTGA ACGAGCAAAC

      1690        1700        1710        1720
ACAGGTTGTG GGTTCGAGTT AGCAAGGGAC GTATAATCTC

      1730        1740        1750        1760
AACTACAATC CATTATGGCG CTTGATGAAA GTCAGCAGTC

      1770        1780        1790        1800
TGATCCATGT AAGGTTCTCT TTTCCTTTAT ATATGCTTCA

      1810        1820        1830        1840
TAATTGAGAA GGAAGACGGA GATTTGAACT TAATAAAGGC

      1850        1860        1870        1880
GAAGATTTGA ACAAAATATT TTGGTATTTC ATTTAAAACT

      1890        1900        1910        1920
TTACCAGTTC TAAGAGTAAA TGATTGGGAT GTGCATGTCC
```

(2r) INFORMATION FOR SEQUENCE I.D. 18
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA (genomic)
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1570 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
        10          20          30          40
TGTGGTGATC GAATTTTCAA TTTTTTTACT GAGTATCTAG

        50          60          70          80
GTTGAGGAAC GTAATTTCAA GCTGCGATCG GCTTTTTCCC

        90         100         110         120
CTGAACGAGC AAACACAGGT TGTGGGTTCG AGTTAGCAAG

       130         140         150         160
GGACGTATAA TCTCAACTAC AATCCATTAT GGCGCTTGAT

       170         180         190         200
GAAAGTCAGC AGTCTGATCC ATTGGTTGTG ATACGCAATG

       210         220         230         240
GAAAGGAGAT CATATTGCAG GCATTCGACT GGGAATCTCA
```

```
        250         260         270         280
TAAACATGAT TGGTGGCTAA ATTTAGATAC GAAAGTTCCT

        290         300         310         320
GATATTGCAA AGTCTGGTTT CACAACTGCT TGGCTGCCTC

        330         340         350         360
CGGTGTGTCA GTCATTGGCT CCTGAAGGTT ACCTTCCACA

        370         380         390         400
GAACCTTTAT TCTCTCAATT CTAAATATGG TTCTGAGGAT

        410         420         430         440
CTCTTAAAAG CTTTACTTAA TAAGATGAAG CAGTACAAAG

        450         460         470         480
TTAGAGCGAT GGCGGACATA GTCATTAACC ACCGTGTTGG

        490         500         510         520
GACTACTCAA GGGCATGGTG GAATGTACAA CCGCTATGAT

        530         540         550         560
GGAATTCCTA TGTCTTGGGA TGAACATGCT ATTACATCTT

        570         580         590         600
GCACTGGTGG AAGGGGTAAC AAAAGCACTG GAGACAACTT

        610         620         630         640
TAATGGAGTT CCAAATATAG ATCATACACA ATCCTTTGTT

        650         660         670         680
CGGAAAGATC TCATTGACTG GATGCGGTGG CTAAGATCCT

        690         700         710         720
CTGTTGGCTT CCAAGATTTT CGTTTTGATT TTGCCAAAGG

        730         740         750         760
TTATGCTTCA AAGTATGTAA AGGAATATAT CGAGGGAGCT

        770         780         790         800
GAGCCAATAT TTGCAGTTGG AGAATACTGG GACACTTGCA

        810         820         830         840
ATTACAAGGG CAGCAATTTG GATTACAACC AAGATAGTCA

        850         860         870         880
CAGGCAAAGA ATCATCAATT GGATTGATGG CGCGGGACAA

        890         900         910         920
CTTTCAACTG CATTCGATTT TACAACAAAA GCAGTCCTTC

        930         940         950         960
AGGAAGCAGT CAAAGGAGAA TTCTGGCGTT TGCGTGACTC
```

```
       970         980         990        1000
TAAGGGGAAG CCCCCAGGAG TTTTAGGATT GTGGCCTTCA

      1010        1020        1030        1040
AGGGCTGTCA CTTTTATTGA TAATCACGAC ACTGGATCAA

      1050        1060        1070        1080
CTCAGGCGCA TTGGCCTTTC CCTTCACGTC ATGTTATGGA

      1090        1100        1110        1120
GGGCTATGCA TACATTCTTA CACACCCAGG GATACCATCA

      1130        1140        1150        1160
GTTTTCTTTG ACCATTTCTA CGAATGGGAT AATTCCATGC

      1170        1180        1190        1200
ATGACCAAAT TGTAAAGCTG ATTGCTATTC GGAGGAATCA

      1210        1220        1230        1240
AGGCATACAC AGCCGTTCAT CTATAAGAAT TCTTGAGGCA

      1250        1260        1270        1280
CAGCCAAACT TATACGCTGC AACCATTGAT GAAAAGGTTA

      1290        1300        1310        1320
GCGTGAAGAT TGGGGACGGA TCATGGAGCC CTGCTGGGAA

      1330        1340        1350        1360
AGAGTGGACT CTCGCGACCA GTGGCCATCG CTATGCAGTC

      1370        1380        1390        1400
TGGCAGAAGT AATCTTACAG CTATTCCGTT ACTTAATATA

      1410        1420        1430        1440
TTAGTAGAAA TATATATGTT TTAAACCCGA GCACCTACTT

      1450        1460        1470        1480
CTAACACTAG ATCCGCCTCT ACAGGCTTGG ATGGAGTGAT

      1490        1500        1510        1520
GAGTTTTTTT TTCCTGTTCA TTAGACATTG CAACATGGGA

      1530        1540        1550        1560
TGTATGTTTT GTTAATAAAA GTGTTCTTGA TCAATGCAAT

      1570
GTAATAAGGG
```

(2s) INFORMATION FOR SEQUENCE I.D. 19
SEQUENCE TYPE: Nucleotide
MOLECULE TYPE: DNA
ORIGINAL SOURCE: *Solanum tuberosum*
SEQUENCE LENGTH: 1570 bp
STRANDEDNESS: Double
TOPOLOGY: Linear
SEQUENCE:

```
        10         20         30         40
ACACCACTAG CTTAAAAGTT AAAAAAATGA CTCATAGATC

        50         60         70         80
CAACTCCTTG CATTAAAGTT CGACGCTAGC CGAAAAAGGG

        90        100        110        120
GACTTGCTCG TTTGTGTCCA ACACCCAAGC TCAATCGTTC

       130        140        150        160
CCTGCATATT AGAGTTGATG TTAGGTAATA CCGCGAACTA

       170        180        190        200
CTTTCAGTCG TCAGACTAGG TAACCAACAC TATGCGTTAC

       210        220        230        240
CTTTCCTCTA GTATAACGTC CGTAAGCTGA CCCTTAGAGT

       250        260        270        280
ATTTGTACTA ACCACCGATT TAAATCTATG CTTTCAAGGA

       290        300        310        320
CTATAACGTT TCAGACCAAA GTGTTGACGA ACCGACGGAG

       330        340        350        360
GCCACACAGT CAGTAACCGA GGACTTCCAA TGGAAGGTGT

       370        380        390        400
CTTGGAAATA AGAGAGTTAA GATTTATACC AAGACTCCTA

       410        420        430        440
GAGAATTTTC GAAATGAATT ATTCTACTTC GTCATGTTTC

       450        460        470        480
AATCTCGCTA CCGCCTGTAT CAGTAATTGG TGGCACAACC

       490        500        510        520
CTGATGAGTT CCCGTACCAC CTTACATGTT GGCGATACTA

       530        540        550        560
CCTTAAGGAT ACAGAACCCT ACTTGTACGA TAATGTAGAA

       570        580        590        600
CGTGACCACC TTCCCCATTG TTTTCGTGAC CTCTGTTGAA
```

```
       610         620         630         640
ATTACCTCAA GGTTTATATC TAGTATGTGT TAGGAAACAA

       650         660         670         680
GCCTTTCTAG AGTAACTGAC CTACGCCACC GATTCTAGGA

       690         700         710         720
GACAACCGAA GGTTCTAAAA GCAAAACTAA AACGGTTTCC

       730         740         750         760
AATACGAAGT TTCATACATT TCCTTATATA GCTCCCTCGA

       770         780         790         800
CTCGGTTATA AACGTCAACC TCTTATGACC CTGTGAACGT

       810         820         830         840
TAATGTTCCC GTCGTTAAAC CTAATGTTGG TTCTATCAGT

       850         860         870         880
GTCCGTTTCT TAGTAGTTAA CCTAACTACC GCGCCCTGTT

       890         900         910         920
GAAAGTTGAC GTAAGCTAAA ATGTTGTTTT CGTCAGGAAG

       930         940         950         960
TCCTTCGTCA GTTTCCTCTT AAGACCGCAA ACGCACTGAG

       970         980         990        1000
ATTCCCCTTC GGGGGTCCTC AAAATCCTAA CACCGGAAGT

      1010        1020        1030        1040
TCCCGACAGT GAAAATAACT ATTAGTGCTG TGACCTAGTT

      1050        1060        1070        1080
GAGTCCGCGT AACCGGAAAG GGAAGTGCAG TACAATACCT

      1090        1100        1110        1120
CCCGATACGT ATGTAAGAAT GTGTGGGTCC CTATGGTAGT

      1130        1140        1150        1160
CAAAAGAAAC TGGTAAAGAT GCTTACCCTA TTAAGGTACG

      1170        1180        1190        1200
TACTGGTTTA ACATTTCGAC TAACGATAAG CCTCCTTAGT

      1210        1220        1230        1240
TCCGTATGTG TCGGCAAGTA GATATTCTTA AGAACTCCGT

      1250        1260        1270        1280
GTCGGTTTGA ATATGCGACG TTGGTAACTA CTTTTCCAAT

      1290        1300        1310        1320
CGCACTTCTA ACCCCTGCCT AGTACCTCGG GACGACCCTT
```

```
      1330        1340        1350        1360
TCTCACCTGA GAGCGCTGGT CACCGGTAGC GATACGTCAG

      1370        1380        1390        1400
ACCGTCTTCA TTAGAATGTC GATAAGGCAA TGAATTATAT

      1410        1420        1430        1440
AATCATCTTT ATATATACAA AATTTGGGCT CGTGGATGAA

      1450        1460        1470        1480
GATTGTGATC TAGGCGGAGA TGTCCGAACC TACCTCACTA

      1490        1500        1510        1520
CTCAAAAAAA AAGGACAAGT AATCTGTAAC GTTGTACCCT

      1530        1540        1550        1560
ACATACAAAA CAATTATTTT CACAAGAACT AGTTACGTTA

      1570
CATTATTCCC
```

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 16, lines 8-13

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution (*including postal code and country*)

23 St Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
26 August 1994	NCIMB 40682

C. ADDITIONAL INDICATIONS (*leave blank if not applicable*) — This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (*if the indications are not for all designated States*)

E. SEPARATE FURNISHING OF INDICATIONS (*leave blank if not applicable*)

The indications listed below will be submitted to the International Bureau later (*specify the general nature of the indications e.g., "Accession Number of Deposit"*)

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Authorized officer R.M. Mandemaker

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Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13*bis*)

A. The indications made below relate to the microorganism referred to in the description on page 16, lines 24-29

B. IDENTIFICATION OF DEPOSIT — Further deposits are identified on an additional sheet ☐

Name of depositary institution

The National Collections of Industrial and Marine Bacteria Limited (NCIMB)

Address of depositary institution *(including postal code and country)*

23 St Machar Drive
Aberdeen
Scotland
AB2 1RY
United Kingdom

Date of deposit	Accession Number
20 October 1994	NCIMB 40691

C. ADDITIONAL INDICATIONS *(leave blank if not applicable)* This information is continued on an additional sheet ☐

In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will be made available until the publication of the mention of the grant of the patent or until the date on which the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *(if the indications are not for all designated States)*

E. SEPARATE FURNISHING OF INDICATIONS *(leave blank if not applicable)*

The indications listed below will be submitted to the International Bureau later *(specify the general nature of the indications e.g., "Accession Number of Deposit")*

For receiving Office use only	For International Bureau use only
☒ This sheet was received with the international application	☐ This sheet was received by the International Bureau on:
Authorized officer R.M. Mandemaker	Authorized officer

Form PCT/RO/134 (July 1992)

CLAIMS

1. A promoter comprising a nucleotide sequence corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof.

2. A promoter comprising a nucleotide sequence corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof but wherein at least a part of the promoter is inactivated.

3. A promoter comprising at least the nucleotide sequence shown as Seq.I.D. No. 1 or a variant, homologue or fragment thereof.

4. A promoter comprising the nucleotide sequence of any of one of the sequences shown as Seq.I.D.No.s 4 – 17 or a variant, homologue or fragment thereof.

5. A promoter comprising a nucleotide sequence corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof, but wherein at least the nucleotide sequence shown as Seq.I.D. No. 1 is inactivated.

6. A promoter comprising a nucleotide sequence corresponding to the 5.5 Kb *EcoR*1 fragment isolated from *Solanum tuberosum*, or a variant, homologue or fragment thereof, but wherein at least any of one of the sequences shown as Seq.I.D.No.s 2 – 16 is inactivated.

7. A construct comprising the promoter according to any one of claims 1 to 6 fused to a GOI.

8. An expression vector comprising the promoter according to any one of claims 1 to 6.

9. A transformation vector comprising the promoter according to any one of claims 1 to 6.

10. A transformed cell or organ comprising the promoter according to any one of claims 1 to 6.

11. A transgenic organism comprising the promoter according to any one of claims 1 to 6 or the invention of any one of claims 7 to 10.

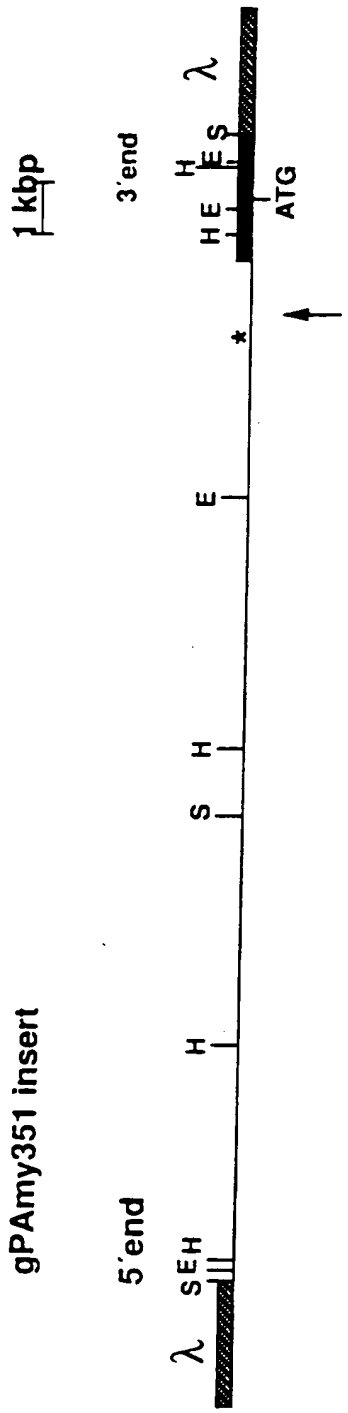
12. A transgenic organism according to claim 11 wherein the plant is a potato plant.

13. Use of the promoter as defined in claim 1 as a cold inducible promoter.

14. A construct comprising the promoter as defined in claim 1 and a nucleotide sequence coding for anti-sense alpha-amylase.

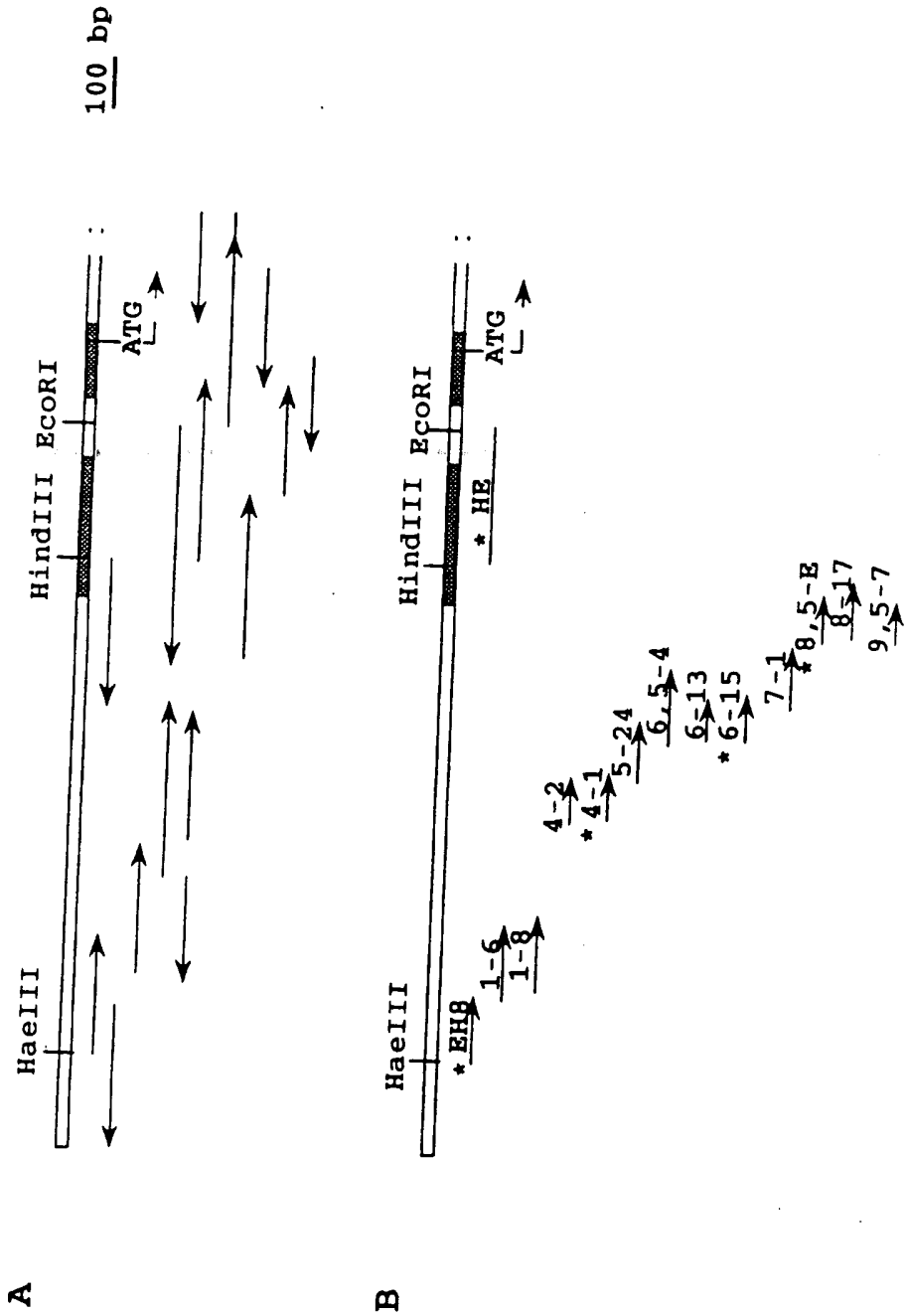
15. Use of a promoter as defined in any one of claims 1 to 6 for expressing a GOI in tuber and/or sprout and/or root and/or stem of a plant.

Fig 1



Restriction enzyme map of the Saturna genomic DNA clone gPAmy351.
The arrow indicates the position of the promoter.
Closed bar: Indicates the position of coding sequences. H: HindIII,
E: EcoRI and S: Sal I. ATG: Initiation codon of the α-amylase coding
sequence. A star marks the position of the 5.5 kb EcoRI fragment.

Fig 2



Sequence map of the α-Amy 3 promoter. The arrows shows the extent of the sequence reactions. The position of the HE fragment is shown in B, together with the 5' sequenced part of the promoter deletion serie. The name of the individual fragments (see also figure 4) is given above the arrows. ATG: Initiation codon of the α-amylase coding sequence. The deletion fragments chosen for functionel analysis are indicated with an asteriks.

FIG.3 3/11

The α-Amy 3 promoter sequence.

- 1734 TCTTTAAGTTGTTTGCTTGATTTTTCTTCTTCAATCTTCTATATTTAATT

- 1684 CGTTTTAGCTTCAAACTTCTTCAATTTTATTTCAATTTAATTCTACAAAA

- 1634 AAAATCTCTATTTAGCACCATTCATAAAATTCATGCTCAAAATGGGCAAA

Hae III

- 1584 CATAAATAATAAATGTGAAGTAAATAATGGATTAAAATATATATTTTTGG

- 1534 GCCTCACATCAACCTTCATAATTCTTGAATGAATGAATGATAGACTTCAT

- 1484 AATTTTTTAACCTATACATATAAGAAAATTGAGAGTAACTCAAATAACAA

- 1434 GTTGTAGTATCACATCTTTACTATTTGATAACATTATGAAGGTGATTATA

- 1384 CATTACGTAACATTTCTTTTAAAAATATGTAAGCAAATTTACTTTTTAAC

- 1334 TTATCATTGATCTTCATGGTTTTGTCATAAATCTCAAAGTTATCATATTT

- 1284 TATATAGCTATTTGAAAGTAATTTTATTTTTACTCATCATTGAGTGATGC

- 1234 TTTTATTATAATACTAGTAAGTTTTATTTATTATTTTCTTTTAGGGGTGA

- 1184 ATTGTATAATATAATAAAAAATATATTTTTAGAAATAATGATTCTTTTAT

- 1134 TATTAAAAAGTTAAGATATTAGATTATTTATGCTTGTATAATAATGAACG

- 1084 AAGTTTTATTTTCTATGAGTTTCATTAATCATGTTTGTAATTATTTCAAA

- 1034 TTTTGATGTATTTTTATAATTTTGTATTATTATATTATTATACTATATTT

- 984 AAAAATTTAAAGATCCATAGGGCTTACGCCCCACGTCAAGAGGCTTGCGC

- 934 CTTTCCCTAAATTAAGTAAAACTCTTCGCCTCATGCCTTACGCCTCCGCC

- 884 TTTTAAAACACTGATTCCTTTCCTCATATAGCTTGAGGCGAAAATATTTA

- 834 ATAAAAACACTTCTTAATTTGTTTATATGTTCAATTGAACATGTCCGTGA

- 784 TTAGAAAATTAAATTAAATTCAATGACAAATTTAATAATTTGACACAAAA

- 734 TTTATGAAAAAAATATCAAAATATAAGAAATATTTTTTTTGAAATGGAT

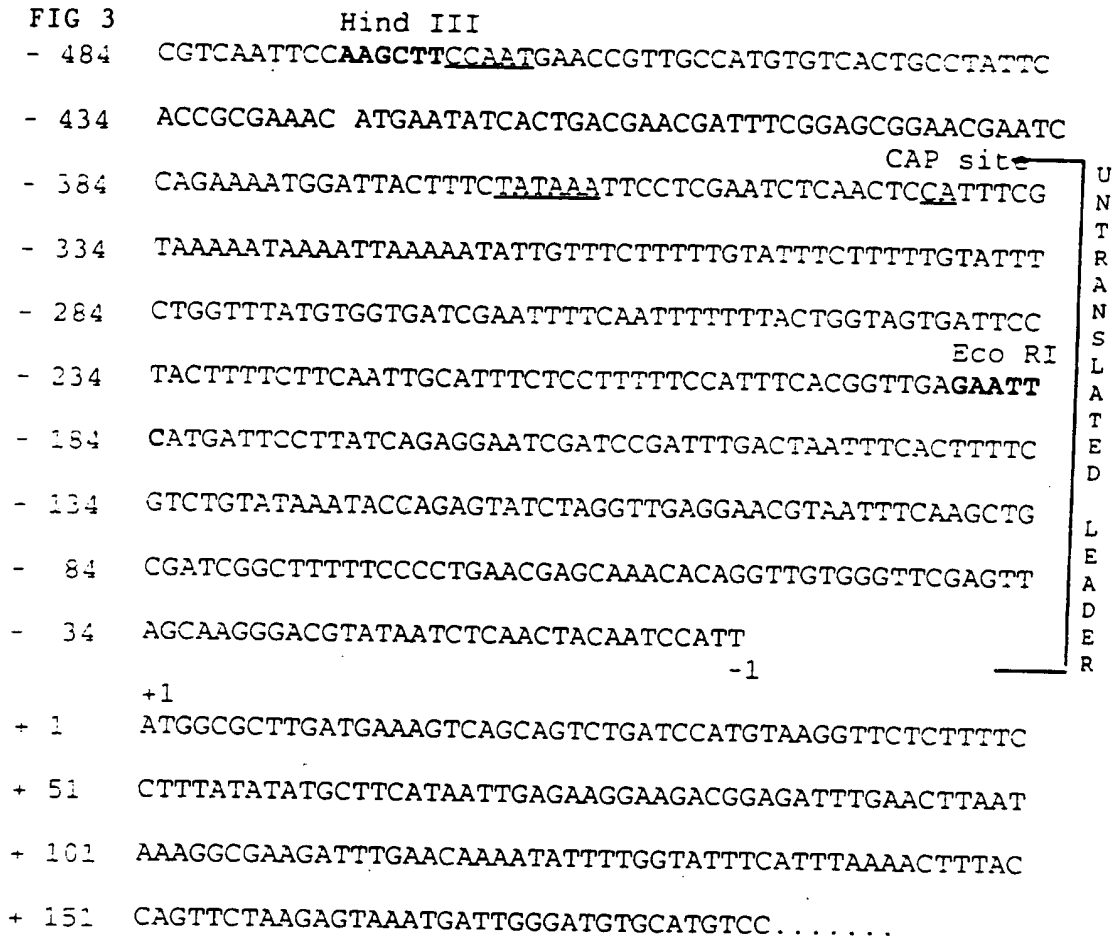
- 684 TAAAAAGAAAAAAAAAACAAATAAATTGAACCGGGATAAGTTGGTTGTTT

- 634 AATTGATTATTGATTATGATCTCAATTTGACATTTTGCGCGATCTTTCGA

- 584 CCTCAATTCGTATGAACTGACACTACGCCAATGGACAGTCGCCGTCGTCA

- 534 CCGCCACCGCACTATTCTCGACGCGTCGTCTATCTCCTCCACCCCACAGC

FIG 3



Restriction sites are bold faced. TATA, CCAAT, and ATG are underlined
The position of the proposed CAP site and the untranslated leader sequence are indicated.

Fig 4

Deletion serie of the α-Amy 3 promoter.

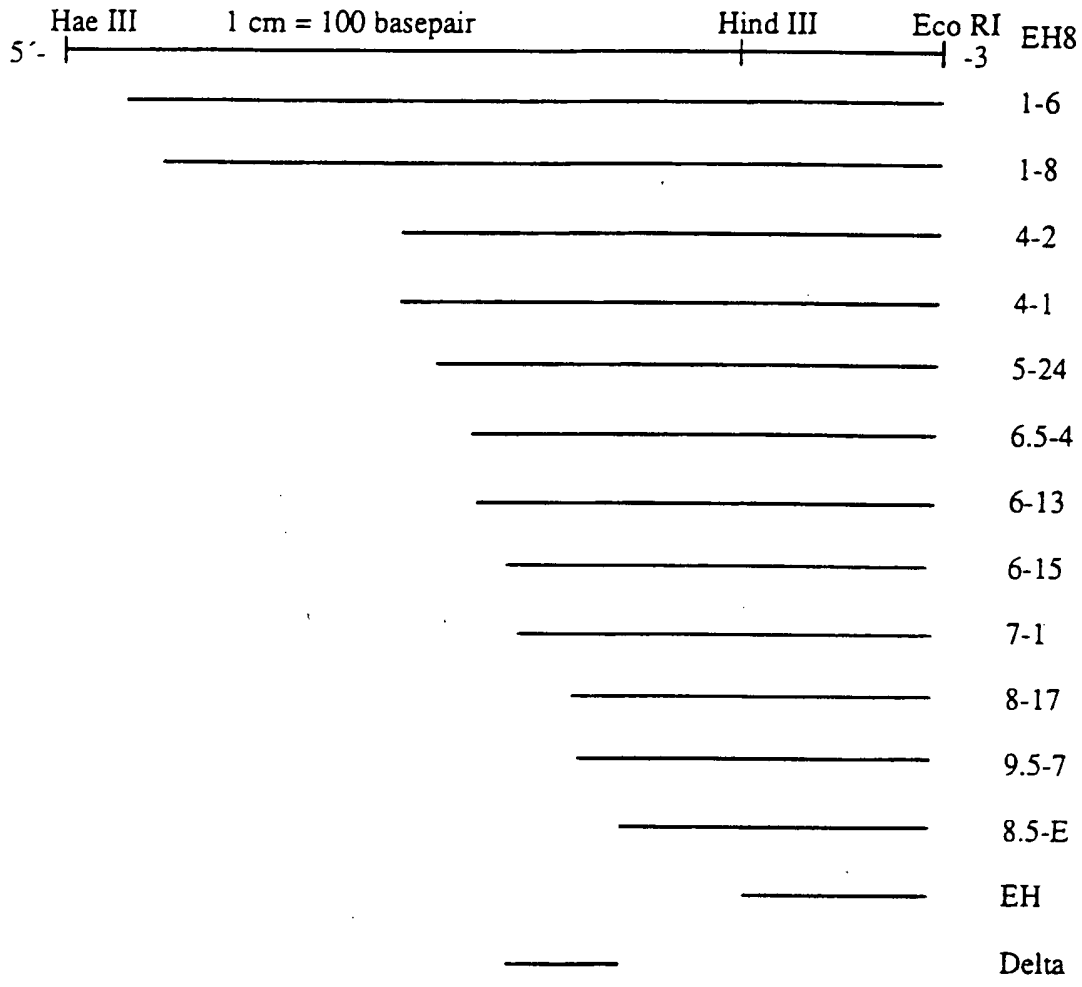


Fig 5

Selected deletions of the α-Amy 3 promoter, for functional analysis.

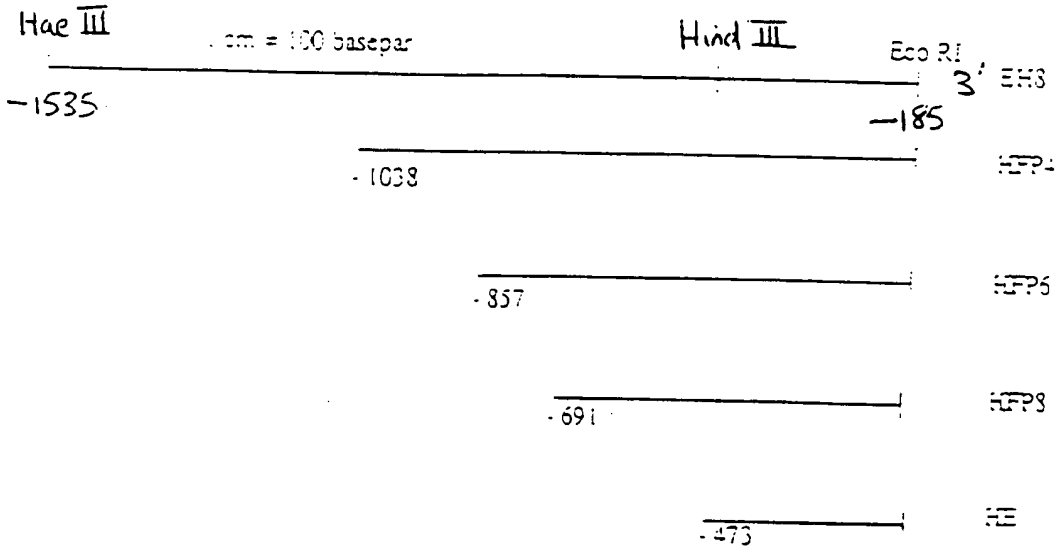


Fig 6

Primer sequences.

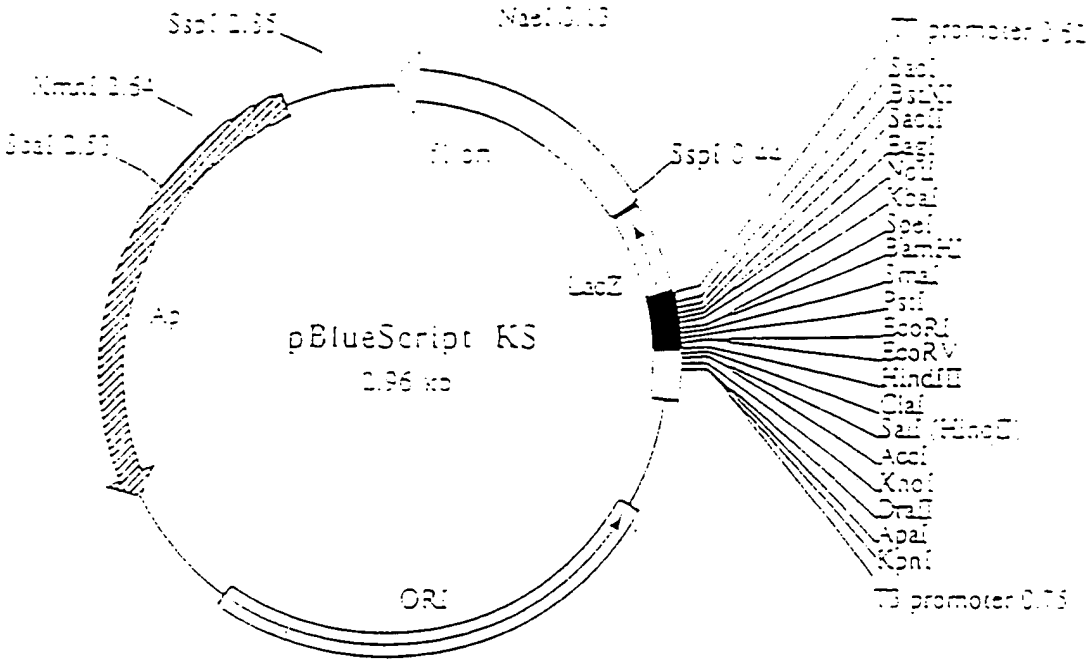
Name	5' - Sequence - 3'
P1	GATAACATTATGAAGGTGAT
P2	GACGGCTGTGGGGTGGAGGAG
P3	CTTGTTATTTGAGTTACTCTC
P7A	CATAAATTTGTGTCAAATTATTAAAT
P8	AGGGGTGAATTGTATAATATAAT
P10	GAAATATTTTTTTTGAAATGGAT
P11	ATTATATTATACAATTCACCCCT

Uni = T7 primer

Rev = T3 primer

# 589	CGCTTTCCCACCAACGCTGAT

Fig 7


pBlueScript KS
2.96 kb
f1 ori
LacZ
Ap
ORI
T promoter
T3 promoter

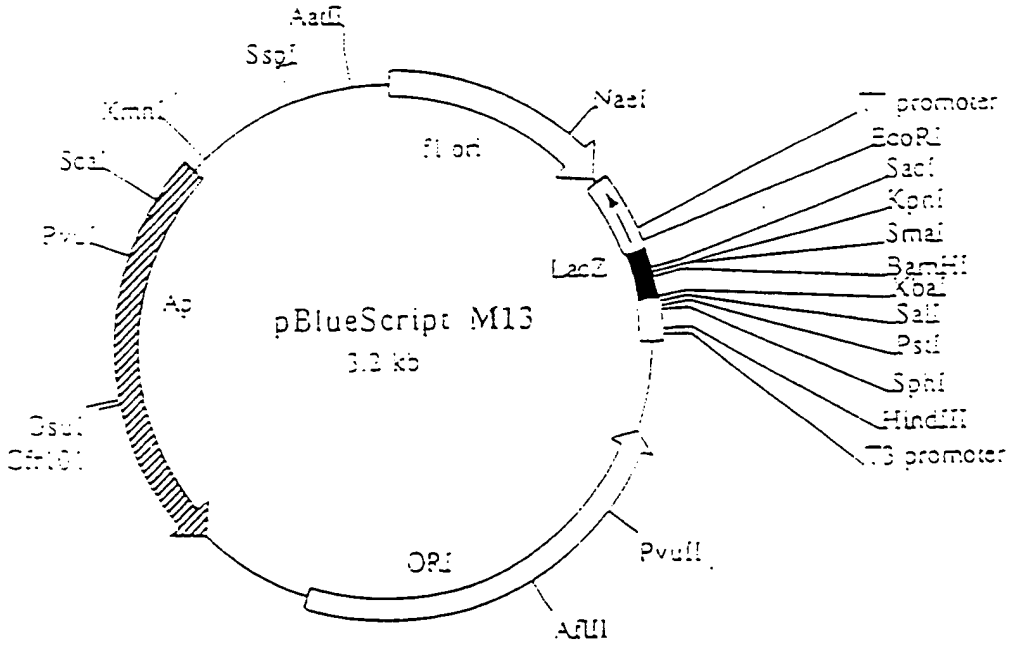

pBlueScript M13
3.2 kb
f1 ori
LacZ
Ap
ORI
T promoter
EcoRI
SacI
KpnI
SmaI
BamHI
XbaI
SalI
PstI
SphI
HindIII
T3 promoter
PvuII
AflII

FIG 8

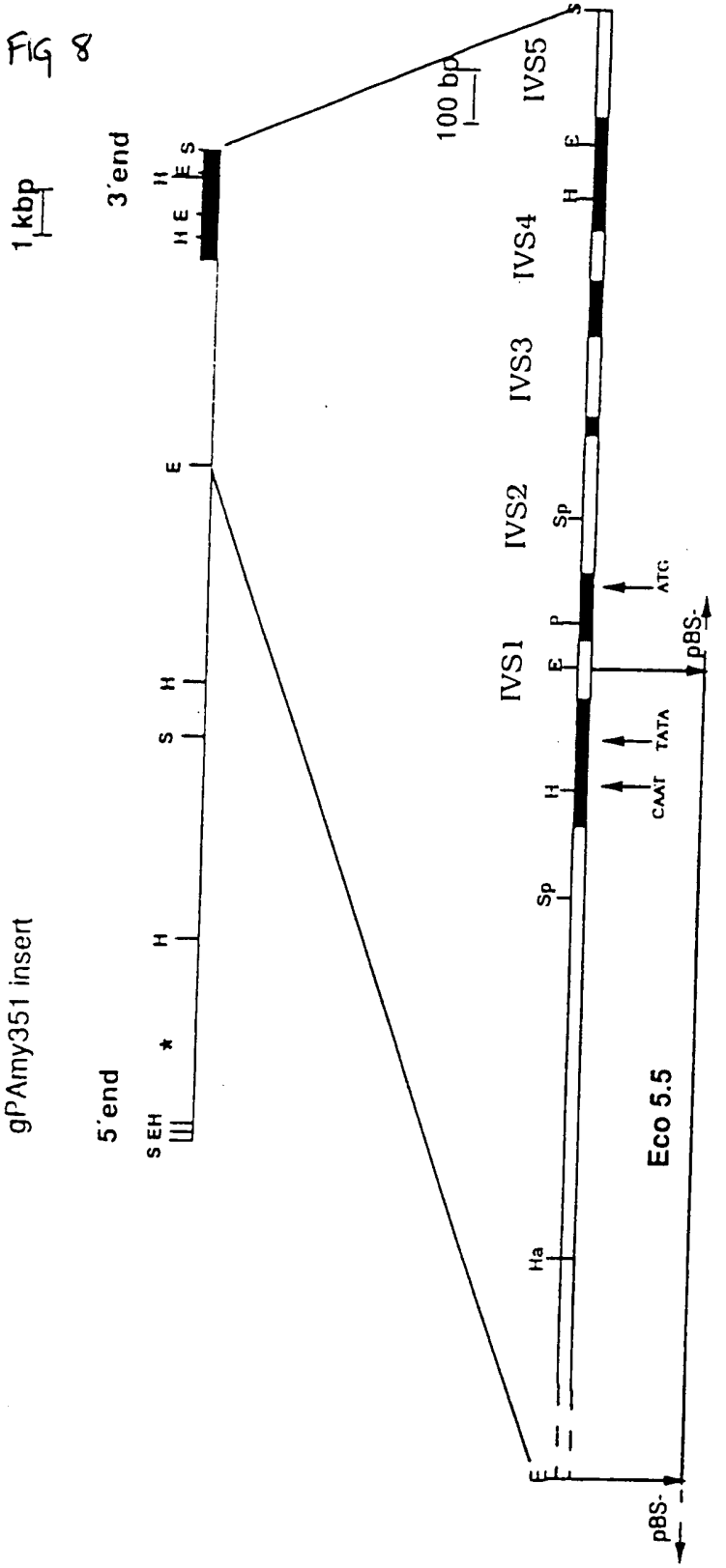
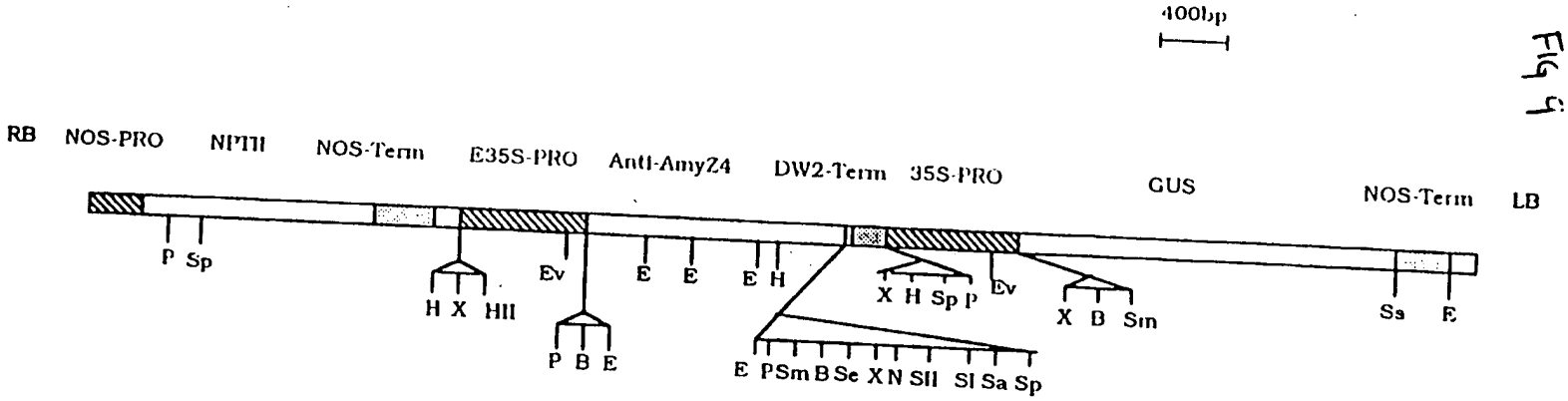

1 kbp
3'end
100 bp
IVS5
IVS4
IVS3
IVS2
IVS1
ATG
TATA
CAAT
pBS-
gPAmy351 insert
5'end
Eco 5.5
pBS-

Fig 9

pJK4



NOS-PRO: Nopaline synthase promoter*

NPTII: Kanamycin resistance*

NOS-Term: Polyadenylation signal from NOS*

E35S-PRO: Double 35S promoter

Anti-AmyZ4: Full length Amy, cloned in antisense direction

DW2-Term:Polyadenylation signal from DW2 (derived from CaMV)

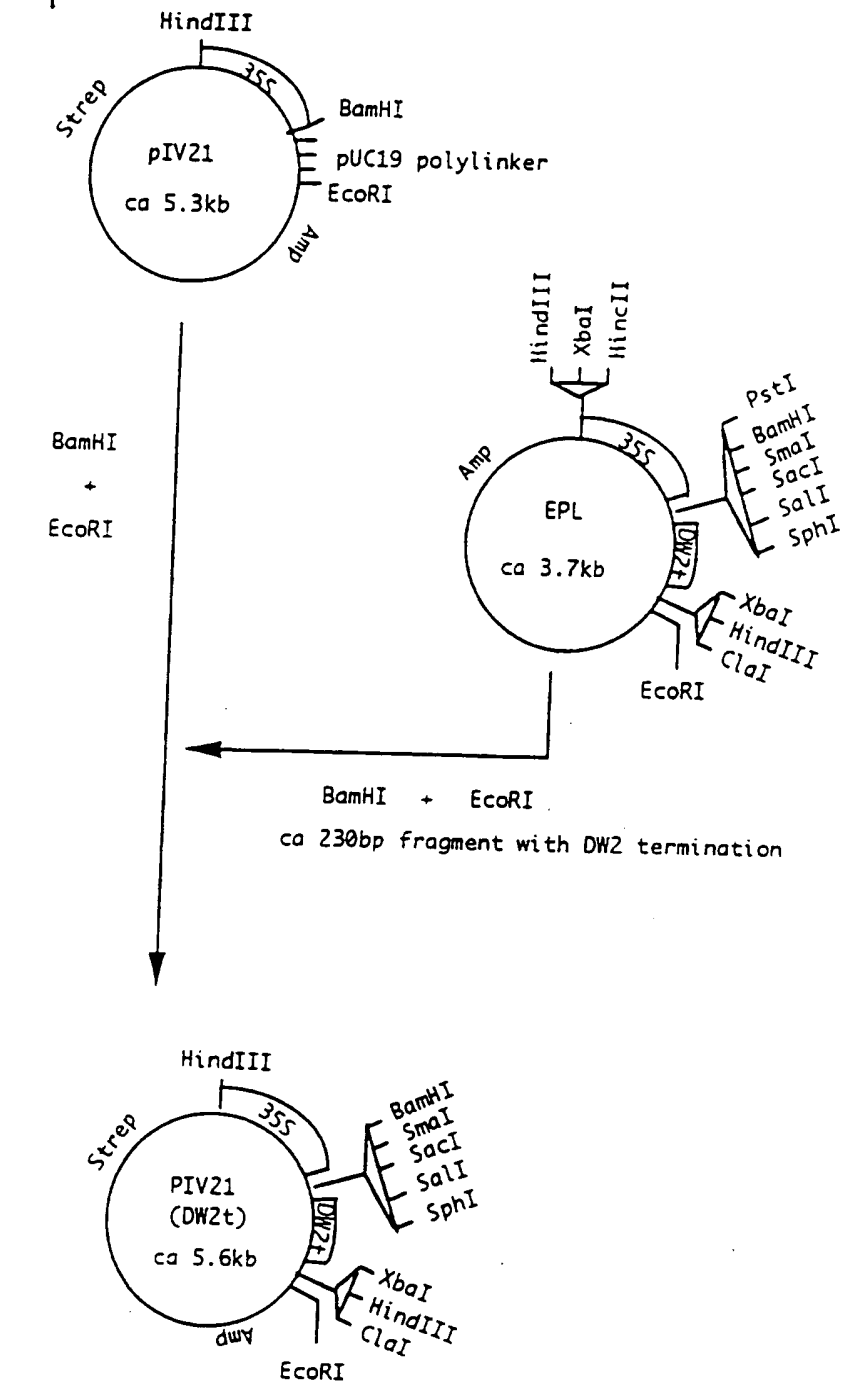
35S-Pro: 35S promoter from CaMV*

GUS: β-Glucuronidase*

*: Part of pBI121

B: BamHI
C: ClaI
E:EcoRI
Ev:EcoRV
H: HindIII
HII: HincII
P: PstI
X: XbaI
Sa: SalI
Se:SpeI
Sm: SmaI
Sp: SphI
Ss: SstI
SI: SacI
SII: SacII
N: Not I

FIG 10


HindIII
35S
Strep
BamHI
pIV21
ca 5.3kb
pUC19 polylinker
EcoRI
Amp
BamHI
+
EcoRI
HindIII
XbaI
HincII
PstI
BamHI
SmaI
SacI
SalI
SphI
Amp
35S
EPL
ca 3.7kb
DW2t
XbaI
HindIII
ClaI
EcoRI
BamHI + EcoRI
ca 230bp fragment with DW2 termination
HindIII
35S
Strep
BamHI
SmaI
SacI
SalI
SphI
PIV21
(DW2t)
ca 5.6kb
DW2t
XbaI
HindIII
ClaI
Amp
EcoRI

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCI/EP 95/02196

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/82 C12N15/56 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 12876 (DANSKE SPRITFABRIKKER) 1 November 1990 see page 98 - page 101; figure 1 see page 110	1,2,4, 7-12,14, 15
A	MOLECULAR AND GENERAL GENETICS, vol. 244, 25 July 1994 pages 127-134, TANIDA, I., ET AL. 'FUNCTIONAL DISSECTION OF A RICE HIGH-PI ALPHA-AMYLASE GENE PROMOTER' see the whole document	1-15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 October 1995	22. 11. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016	Maddox, A

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

.nformation on patent family members

Inte nal Application No
PCI/EP 95/02196

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9012876	01-11-90	AU-B-	638248	24-06-93
		AU-B-	5531890	16-11-90
		DE-D-	69008172	19-05-94
		DE-T-	69008172	20-10-94
		EP-A-	0470145	12-02-92
		ES-T-	2053189	16-07-94
		IE-B-	63067	22-03-95

INTERNATIONAL SEARCH REPORT

I. ational application No.
PCT/EP 95/02196

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Please see annex!

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

ANNEX INCOMPLETE SEARCH SA112268(PCT/EP9502196)

The ISA is of the opinion that due to the parameters and wording (variant, homologue or fragment) used to define the sequences of claims 1-15 that the scope of the claims is not adequately defined and therefore are not considered to be clear and concise within the meaning of article 6 PCT, and for this reason the ISA notifies in accordance with article 17 (2)(a)(ii)PCT that the search for the claims 1-15 has been restricted to the sequences as represented by sequence I.D. numbers 1-17 and the use and embodiments of said sequence I.D.'s as defined by claims 7-15.